IMPACT OF CROSS-FOSTERING ON THE INTESTINAL MICROBIOME AND MUCOSAL IMMUNE GENE EXPRESSION IN NEONATAL PIGS

BY

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THESIS

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ABSTRACT

Colostrum is vital to the newborn pig. Hence, cross-fostering is employed to equalize the number of piglet between litters ensuring colostrum intake for their survival and growth. However, little is known about the impact of cross-fostering on the intestinal microbiome and mucosal immune gene expression of the neonatal pig. Twenty-four piglets were enrolled in the study to determine the influence of maternal microbial communities and to establish a baseline for mucosal immune gene expression in young pigs reared in cross-fostering model given high quality colostrum from birth dam or foster dam upon birth. Piglets were randomly assigned to 1 of 3 treatments according to colostrum source and postcolostral milk feeding for 21 days, as follow: treatment 1 (n = 8), received colostrum and post-colostral milk feeding from their own dam; treatment 2 (n = 8), received colostrum from foster dam and returned to their own dam for post-colostral milk feeding; and treatment 3 (n = 8), received colostrum and post-colostral milk feeding from foster dam. DNA was extracted from nasal, fecal, and gastrointestinal (GI) tract of the piglets and from colostrum, vaginal, and fecal samples of the sows. Tissues from intestinal mucosa in jejunum, ileum, colon, peyer's patches, and associated lymph nodes were utilized. Quantitative real-time PCR analysis was performed to quantify the expression of toll-like receptors (TLR) 2, 4, and 10, tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), and interleukin (IL) 4 and 10. Discriminant analysis revealed that bacterial communities varied with biogeographical location in the GI tract, with colon being the most diverse section. *Firmicutes* and *Bacteroidetes* were the dominant phyla in the GI tract of the young pig. Bacterial communities in both maternal colostrum and vaginal samples were significantly associated with those present in the GI tract, feces, and nasal passage of piglets. Treatment did not affect bacterial communities present in the piglet GI tract, however, the bacterial communities present

in piglet fecal and nasal samples changed over time. The mRNA expression of TLRs and inflammatory cytokines changed ($P < 0.05$) with biogeographical location in the GI tract. Higher mRNA expression of TLRs and inflammatory cytokines was observed in ileum, ileum lymph nodes and peyer's patches tissues. Although cross-fostering did not impact microbial communities in the piglet, this study suggests an impact of colostrum and maternal influence on the development of the microbiome of the piglet. This study revealed novel information about the distribution and expression patterns of TLRs and inflammatory cytokines in the GI tract of the young pig.

Keywords: cross-fostering, colostrum, piglet, microbiome, gastrointestinal, gene expression

DEDICATION

This thesis is dedicated to the memory of my beloved mother Reyna Maradiaga. I miss her every day, but I am glad she left happy knowing I was pursuing my dreams. Great appreciation is extended to my sister and my husband for their support and encouragement.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure

LIST OF ABBREVIATIONS

16S rRNA	16 S ribosomal RNA
AMPs	antimicrobial peptides
ARISA	automated ribosomal intergenic spacer analysis
DGGE	denaturing gel electrophoresis
FISH	fluorescent in situ hybridization
GALT	gut associated lymphoid tissues
GI	gastrointestinal
IFNγ	interferon gamma
IL	interleukin
MAMPS	microbial–associated molecular patterns
NF-KB	nuclear factor kappa-light-chain-enhancer of activated β cells
NGS	next generation sequencing
NIH	national institute of health
OTU	operational taxonomical unit
PCoA	principle coordinate Analysis
PRR	pattern recognition receptors
QIIME	quantitative Insights into Microbial Ecology
RDP	ribosomal database Project
sIgA	secretory IgA
TLR	toll-like receptors
TNFα	tumor necrosis factor alpha
TRFLP	terminal restriction fragment length polymorphism

CHAPTER 1

LITERATURE REVIEW

Importance of Mucosal Microbial Communities in the Gastrointestinal Tract of the Neonatal Pig

Fifty percent of preweaning mortality in piglets can be attributed to gastrointestinal and respiratory infections acquired during the first 72 hours after birth. The high levels of morbidity and mortality associated with infectious disease during this fragile stage of the production cycle is a serious cause of economic loss and welfare concern to the swine industry. In addition, infectious disease during the first weeks of life is an important driver of antimicrobial use in the swine production life cycle. In view of increasing concerns regarding antimicrobial residues in livestock-based food, and their potential role in the development of antimicrobial resistance, there is a growing interest in new strategies that could help increase host resilience, and so lower the frequency of disease during this vulnerable developmental period.

The role of colostrum in protection against neonatal infectious disease is well established. Cross-fostering is a management practice that is commonly used to maximize colostrum intake in the piglets. This entails the transfer of one or more piglets between litters, soon after birth, to equalize litter size and weights (Kirkden et al., 2013). The intake of an adequate volume of good quality colostrum during post-partum period is extremely beneficial to the health and development of the newborn piglet. Colostrum provides a rich source of nutrients for sustenance and growth, promotes epithelial health through the provision of cytokines and growth factors, and most importantly, supplies a wide range of soluble and cellular immune factors that provide local and systemic protection against infectious disease. Under optimal conditions, the duration

of immunological defense provided by the exogenous, maternally-derived, colostral immune factors, coincides with the development of the piglet's own active, endogenous immune capability. The development of an adequate, effective and primed mucosal immune system involves the coordinated and measured exposure of the intestinal tract to environmental antigens. Recent studies have demonstrated a direct link between the developing gastrointestinal microbiota, and the health and disease susceptibility of growing pigs (Mann et al., 2014). The role of the gastrointestinal microbiota in nutrient utilization, intestinal permeability and immune development has been clearly demonstrated (Round and Mazmanian, 2009; Geuking et al., 2011; Ohnmacht et al., 2011; Schokker et al., 2014). While these previous studies provide strong evidence that the gastrointestinal microbiota contribute significantly to gut health and immunological fortitude (Collado et al., 2012), few studies have described the internal host and external management factors that might contribute to the development of a balanced microbiota and therefore establish a baseline between host immunity and microbiota. It is important to appreciate that the gastrointestinal tract is a complex ecosystem (Gordon and Pesti, 1971) comprised of a series of unique, anatomically-, and physiologically-distinct compartments, each of which represents a unique ecological niche. In view of the biogeographical complexity of the gastrointestinal microbial community, and the fact that microbial community structure varies with time, environmental conditions and location (Dubos et al., 2005), it is interesting that many studies have focused on the fecal microbiota. While the colon harbors some of the most diverse microbial communities, it is evident that fecal samples are unlikely to be representative of the entire gastrointestinal tract, and it is well known that there is variation in microbial community structure along the gastrointestinal tract. Therefore further studies that provide an integrated

assessment of the microbial communities in different biogeographical locations along the alimentary tract are necessary.

Analysis of Microbial Communities: 16SrRNA Sequencing

The rapid growth in our understanding of the complexity and importance of the host-microbiota relationship has been driven by the breathtaking advances in the availability and affordability of new, high speed, high throughput nucleotide sequencing technologies, and the development of bioinformatic tools that can be used to characterize and analyze large sequence data sets. Early studies of microbial communities relied on anaerobic culture-based techniques, which involved the isolation and growth of specific bacterial colonies, and the subsequent identification of strains by examining colony morphology, microscopic appearance, and fermentative capacities. While these traditional methods were successful in identifying a moderate number of bacterial species in the gastrointestinal tract, the growth and cultivation properties of some bacterial species meant that these approaches were limited in their capacity to characterize complex ecosystems (Handelsman, 2004; Dave et al., 2012). For instance, many culture methods favor the selective growth of some microorganisms over others, and thereby alter the natural composition of a community. Another drawback of culture-based techniques is the laborious and time-consuming nature of the procedures. This confers a practical limitation on the number of samples that can be efficiently processed, and so precludes the application of these techniques in large scale population studies (Leser et al., 2002).

The first generation of culture-independent technologies was based on the amplification, fragmentation, denaturation, hybridization or sequence/size differentiation of strain-specific

DNA fragments. They included denaturing gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (TRFLP), automated ribosomal intergenic spacer analysis (ARISA), and fluorescent in situ hybridization (FISH), and were commonly referred to as "fingerprinting" techniques. Although fingerprinting techniques brought a unique method and depth of microbial identification, they were criticized for their inability to provide species-level identification, and for their variable threshold limitations in detecting less abundant bacterial taxa. In addition, these methods continued to be laborious, and relatively expensive (Bent et al., 2007).

One extremely important development in the evolution of molecular-based approaches to bacterial identification, was the recognition of the inter-species variability of the 16S ribosomal RNA (rRNA) gene family (Woese, 1977). These genes encodes the 16S rRNA of the small ribosomal subunit, are present in all prokaryotes and archaea (Kim and Isaacson, 2015), and exhibit taxa-specific variation in their genomic sequence. For this reason, 16S rRNA gene sequences have become the most commonly used molecular markers for studying bacterial phylogeny and taxonomy, and can be used to provide even genus and species identification (Janda and Abbott, 2007). The universal presence of this gene family across prokaryotes means that 16S rRNA gene sequencing has also been useful for identifying unusual, non-cultivable, and phenotypically or biochemically indistinguishable strains. By incorporating a sequence-specific amplification step, investigators have been able to apply this approach to fastidious microbes that cannot be propagated in culture, and to help identify those that may be present in low abundance in a sample. The application of 16S rRNA gene sequencing to bacterial identification has also significantly reduced time and labor and has offered great opportunities for scalability in experimental design. As with all research tools, 16S rRNA gene sequencing has some significant

drawbacks when compared to culture-based techniques. For instance, in its simplest form the technique is non-quantitative, and cannot differentiate between the presence of live organisms and naked DNA. The broad sequencing approach also generates a large amount of data that requires detailed biostatistical analysis to foster reliable interpretation. Thus, while 16S rRNA sequencing-based approaches have certainly provided new insight in to the complexities of gastrointestinal microbiology, before using these techniques it is important to understand the caveats and potential drawbacks so that the results can lead to meaningful and accurate conclusions.

Optimizing Quality and Reliability in the Study of Microbial Communities

In any experimental system, investigator confidence in the study outcomes is ultimately determined by the reliability of the tools and the reproducibility of the results. The 16S rRNA gene sequencing-based techniques are often applied to the analysis of complex microbial communities in diverse environments, each of which can impact optimal sample processing, effective target amplification, and ultimately sequencing fidelity. To avoid selective isolation and amplification of DNA from certain microbes, and to ensure that the nucleotide sequences generated during the process are representative of the taxa present in the original sample, careful attention must be given to sample processing and to primer design. Proper sample collection and complete DNA isolation are crucial determinants of the reliability of downstream procedures.

Firstly, in view of the universal distribution of microbial nucleic acids in the environment, samples must be collected swiftly and hygienically. Following collection, careful and appropriate handling of the sample is essential for maintaining DNA integrity, and to prevent

the growth of opportunistic contaminants. For instance, samples can be collected in to a solution containing nuclease inhibitors prior to subsequent sample dilution and DNA extraction. If the DNA extraction cannot be performed immediately, samples must be frozen on dry ice and stored at −80 °C pending further processing (Highlander, 2012).

Secondly, DNA must be isolated before attempting any downstream amplification or analysis. Currently, there are many commercially-available kits for DNA isolation, most of which utilize similar processes and protocols. An initial lysis of host and microbial cells is facilitated by chemical, enzymatic, and/or mechanical disruption of cell membranes. The efficiency and completeness of this lysis step has a significant impact in determining the extent and proportion of microbes that are identified in a particular microbial community (Highlander, 2012). Traditionally, chloroform-based centrifugation methods were used to extract DNA, but there has been a more recent preference for the use of strong detergents in combination with physical disruption. For example, MO BIO Laboratories, Inc. (Carlsbad, CA) Power Soil® DNA isolation kits use Sodium dodecyl sulfate (SDS) as a strong detergent, and a bead beating method that lyses cells by collision of tiny glass beads with the sample. Alternatively, some approaches [e.g. Qiagen (Valencia, CA) QIAamp DNA Stool Kit)] use high temperature incubation with a detergent, followed by enzymatic treatment of the sample. Isolated DNA is subsequently recovered from the lysate by fractionation on a spin column, or by ethanol precipitation. The final steps of any of these bench-top kits, is clean, purified DNA that is ready for PCR amplification and further analysis.

The efficiency of amplicon detection and amplification is another key step in determining the quality of data that is generated by 16S rRNA gene sequencing-based techniques. Both of these steps are impacted by the effectiveness of primer selection or design. The 16S rRNA gene

is present across all bacterial taxa, and contains a combination of highly conserved regions, interspersed with nine (V1-V9) hypervariable regions. The hypervariable regions contain the genus- and species-specific sequences and so are the main target sites for 16S rRNA gene-sequence based approaches. By designing PCR-primer sets that recognize unique sequences within the hypervariable regions, amplicons from specific taxa of bacteria can be identified. Alternatively, by designing universal primers complementary to the highly conserved sequences that flank the hypervariable regions, all of the 16S rRNA genes present across different microbial domains, such as archaea or bacteria, will be amplified from a mixed microbial sample (Ludwig et al., 1994). Some universal primers are more competent than others, and certain longer hypervariable regions of the 16S rRNA gene reveal more information than others (Liu et al., 2007; Schloss and Eisen, 2010). For instance, hypervariable regions V2 and V4 are often used because of their low error rates (Liu et al., 2007), and regions V3-V4 are known to offer greater taxonomical precision (Claesson et al., 2009). On the other hand, region V6 is rarely used, because it has been shown to produce confounding data regarding certain major phyla in the gut. Overall, there is no general consensus as to which 16S rRNA hypervariable region offers the most advantages. As a result, multiple regions are often used to help capture the full range of diversity of microorganisms in a sample (Sogin et al., 2006; Highlander, 2012).

For many years full-length sequence analysis of cloned 16S rRNA genes were considered the gold standard for bacterial and archaeal classifications (Highlander, 2012). More recently, the amplification of partial hypervariable regions of the 16Sr RNA gene has been shown to provide comparable information to that described for full length sequence analysis at the genus level or higher (Kim, 2011). Modern DNA sequencing techniques, such as those termed high throughput, or next generation sequencing (NGS), have revolutionized the time efficiency and depth

effectiveness of microbial sequencing. For instance, NGS can generate reads greater than 1,000 nucleotides. This allows for a more complete coverage of sequences, and thus exposes a greater breadth and diversity of microbes in a particular sample. The capacity and power of these new technologies has also fostered a decrease in time and cost for DNA sequencing. Currently, various platforms are available including the Ilumina HISeq 2000 and the Roche 454 FLX Titanium. They rely on pyrosequencing techniques commonly known as "multiplexing", a term referred to the addition of a unique tag or barcode that would identify the nucleotides by the amplitude of light emissions (Wooley et al., 2010). Barcoding amplicons increases the efficiency and decreases the cost of multiple microbial communities being sequenced simultaneously (Liu et al., 2007). While the use of NGS technology has certainly improved our ability to accurately describe and characterize microbial communities, its application still carries a number of potential pitfalls, the detailed discussion of which are beyond the scope of this paper. It is important to recognize that although NGS technology makes sequencing costs affordable, amplicon preparation and library construction remains laborious and tedious. It is anticipated that the automatization of these procedures will gradually diminish expenses and processing time (Highlander, 2012).

Bioinformatics Analysis and Taxonomical Classification

Once the 16S rRNA sequence data has been generated, computational processing and adjustment of sequences remains a tremendous challenge, especially for researchers unacquainted with biostatical analysis. Several steps need to be taken to minimize errors in calculation and classification of reads. Quality filtering or "cleaning" of raw data is a crucial

element in the process of obtaining reliable results. Auspiciously, the computational industry have developed unique and convenient software to support researchers that lack programming or bioinformatics experience. Open-source bioinformatics pipelines are available that can assist in the complete or partial 16S rRNA analysis. MG-RAST, Mothur and Qiime (Caporaso et al., 2010; Schloss and Eisen, 2010), are frequently used for quality checks and "trimming" of reads. Moreover, the detection and elimination of artifacts created during PCR amplification of 16S genes known as "chimeras" is essential to reliable representation of diversity. According to Haas et al. (2011), chimeras were responsible for the majority of problematic sequences and can be removed using tools such as Chimera Slayer (Haas et al., 2011), or UCHIME (Edgar et al., 2011). It is important to eliminate chimeras since these are incline to magnify species abundance and are prone to errors in classification. Undoubtedly the future development of user friendly pipelines will help scientists and student researchers expand their knowledge and generate meaningful information. Trimmed and chimera free sequences are submitted to databases and aligned against known sequences for taxonomical classification. The most common databases are, Greengenes (Schloss, 2013), BLAST, SILVA (Pfeiffer et al., 2014) and the Ribosomal Database Project RDP (Looft et al., 2012), pipelines like Mothur and Qiime can accomplish various tasks, quality filtering, clustering of operational taxonomical units (OTU's), taxonomic assignments and calculations of bacterial diversity in one package. Diversity estimates allow for the detection of minor changes in microbial composition within one particular community and between communities.

Diversity Measurements

Alpha-diversity determines the diversity of microbes within a community; it exposes the richness denoted as the amount of OTUs in a sample. Common nonparametric measures of alpha diversity are the Chao1 estimator (Chao, 1984) based primarily on microbial richness and Shannon Index, grounded on the richness and closeness of bacterial groups in a community. Diversity between bacterial communities is known as beta-diversity (Whittaker, 1972), it can be determined using similarity indices; distance matrices are generated and later visualized as clusters (Highlander, 2012). Alternatively, Unique Fraction metric or UniFrac is a beta diversity measurement commonly used (Caporaso et al., 2010), it takes into consideration the different measures of similarity between sequences and calculates distance matrices than can be later used in PCoA (Principle Coordinate analysis). On the other hand, the Simpson Index, Bray Curtis Index and Morisita-Horn have similar ratios used to determine the number of shared species in relation to the entire population. In addition, other visualization aids such as hierarchical clustering and phylogenetic trees are commonly used to help describe beta-diversity (Highlander, 2012). Approaching the challenges and reasonably scrutinizing the amount data generated by 16S rRNA technology is critical to create meaningful information that will benefit production on the development of management strategies that will help shape up the developing microbiome of an individual.

Current State of Knowledge: Gastrointestinal Microbiota of the Pig

Considerable amount of literature has been published in humans on host-microbe interaction. The National Institute of Health (NIH) developed "The Human Microbiome Project" (Kim et al., 2011) that with the help of next generation sequencing technology (NGS) gave the scientific community a better understanding of the relationship between the myriad of species

colonizing the human and the host immune system. Early colonizers once considered pathogens are now given a second chance, proving to be critical constituents and influencers of the intestine immune status (Marchesi, 2011; Collado et al., 2012).With these advances, new bacterial strains are revealed to coexist in places once considered “sterile” suggesting a deeper relationship of these microbes with the host (Reid et al., 2015). Recent research suggest that microbiota composition is among the most important factors influencing the development of diseases such as intestinal complications, cancer, obesity, asthma and diabetes just to mention a few (Isolauri, 2012; Voreades et al., 2014). This early bacterial establishment is fundamental to the prevention of health problems and regulation of the immune system (Collado et al., 2012; Hansen et al., 2012). In the same way microorganisms administered as probiotics have significantly improved quality of life (Reid et al., 2015), suggesting the potential use of microorganisms as health promoters. Throughout this review several examples regarding human microbiota and its relationship with the host will be exemplified. Although most of the current research is related to humans, several studies have documented the similarities between pigs and humans providing new insights into the pig’s relationship with bacterial communities and the impact of these microbial communities on animal performance.

Anatomically pigs are similar to humans and for many years pigs have been used as model for human diseases (Xian et al., 2014; Zhao et al., 2015) as they share similar intestinal bacterial composition and dynamics from birth to maturity (Konstantinov et al., 2006; Thompson et al., 2008). At birth, the intestinal tract of newborn mammals is exposed to a cocktail of microorganisms; this freshly established microbiota will colonize and stabilize the undefined GI tract. In humans, the passage of antibodies from mother to offspring provides the immunological protection needed to deal with these new invaders (Rindsjö et al., 2010; Esposito et al., 2012).

The main disadvantage with pigs is they are born agammaglobulinemic, that is, there is no vertical transfer of immunoglobulins during gestation. Consequently, newborn pigs depend entirely on the sow's colostrum for nutrient acquisition and immune protection (Decaluwé et al., 2014). For this reason piglets are more susceptible to diseases, especially enteric and respiratory, during their first days of life (Tuchscherer et al., 2000) which in return bring great economic loss to the swine industry. One of the greatest challenges for the swine industry has been developing strategies to improve piglet's performance and overall health. Most of these strategies involve the use of antibiotics in the diets as growth promoters and diarrhea reduction agents (Levesque et al., 2014; Kim and Isaacson, 2015). Little is known about the effects of antibiotics in the overall microbiota composition. However, recent welfare concerns regarding antibiotic residuals in food and antibiotic resistance has led to exploration of other areas of host-disease interaction. Intestinal microbiota in pigs can be influenced by many factors including environment, age, stress, and nutrition (Guo et al., 2008; Thompson et al., 2008; Levesque et al., 2014); and thus, the microbiota profile accomplished by maturity will determine the well-being of the pig. A healthy microbiome is key to the individual health and changes in microbial composition can lead to disease (Voreades et al., 2014). Therefore, determining a healthy microbiome in the pig's intestine and understanding its relationship with the host can lead to the development of better management practices in swine husbandry.

Early Bacterial Inheritance and Immunological Contribution

The exact origin of the mammalian intestinal microbiota is still unknown. According to Stark and Lee (1982), the mother's microbiota is responsible for colonizing the offspring

gastrointestinal tract during the first years of life, thereafter, this microbiota is influenced by environmental factors and diet (Collado et al., 2012). Likewise Kim and Isaacson (2015) suggested, that initial microbiota is acquired through birth where the piglet is continuously exposed to the sow's mucosal bacterial communities. In fact, recent studies have found microbes to be present in placental and fetal tissues (Reid et al., 2015) suggesting the uterus is not sterile after all, and microbes are metabolically and functionally important to the host. In a similar way, recent studies suggest that breastmilk provides the offspring with oligosaccharides that modify bacterial composition (Donovan et al., 2012). Breastmilk also provides the newborn with key microbes beneficial for immune modulation, enhancement of epithelial integrity and absorption of nutrients (Collado et al., 2009; Fernández et al., 2013). Although it is still unknown how these microbes are capable of colonizing mammary glands, Martín et al. (2004) proposed a mechanism of regurgitation in the mother's gut that allows these microbes to travel from the gut to the fetus and mammary glands. Additionally, Rescigno et al. (2001) and Macpherson and Uhr (2004) clarified that antigen presenting cells stimulate mucosal immunity by holding live bacteria inside and presenting it to the immune system, providing an opportunity for microbes to migrate.

Breastmilk not only provides the individual with oligosaccharides necessary for growth, but also transmits microbes that are necessary to stimulate an immune response. Breastmilk has been discovered to contain *Bifidobacteria, Lactobacilli and Staphylococci* species (Martín et al., 2003; Thompson et al., 2008; Isolauri, 2012; Fernández et al., 2013). Similarly, Mach et al. (2015) reported one hundred and eighty-two operational taxonomical units (OTUs) where shared between sow and piglet supporting the idea of bacterial strains passed down to the offspring via breastmilk. Interestingly, diet fortification with *Lactobacillus* in children generated a 46% decrease in intestinal diseases (Fernández et al., 2013). In a study Schokker et al. (2014) assigned

twenty piglets to three groups to determine whether stress and antibiotic usage had an effect on jejunal microbial composition and immune response. In their findings, the control group revealed higher immune response compared to the stress and antibiotic group. The authors correlated this response to a *Lactobacillus* increase. The other groups, antibiotic and stress administration, showed a significant reduction in *Lactobacillus* and immune response. Schokker et al. (2014) concluded that *Lactobacillus* is necessary to effectively stimulate the immune system and that antibiotic usage eliminates the initial pathogens and *Lactobacillus* strains, and thus, weakens the immune response. In a similar study Mach et al. (2015), found a strong relationship between microbiota and the immune response. In their findings, the presence of *Prevotella,* belonging to the phylum *Bacteroidetes,* was positively correlated with luminal secretory IgA, indicating a strong influence of gut microbiota in mucosal immunity. Xian et al. (2014) studied the effects of cross-fostering on cecal microbiota, finding better growth rate, and decreased *Bacteroidetes* in fostered piglets. Xian et al. (2014) concluded that artificial milk in fostered piglets changed microbial composition; however, the weight gain of cross-fostered piglets cannot be attributed to the microbial composition but to the nutritional richness of the artificial milk. This idea of milk source having an impact on gut microbiota is supported by the studies of Harmsen et al. (2000), Poroyko et al. (2010), and Li et al. (2012). Li et al. (2012) found an increase in *Bifidobacterium* and *Clostridium* in piglets suckling directly from the sow and an increase in *Bacteroides vulgatus* in piglets fed with formula. Surprisingly, Harmsen found similar results in fecal microbiota of children fed with formula compared to breast-milk. Breast-fed children have higher amount of *Bifidobacteria*, whereas *Bacteroides* in bottle-fed children equalized the amount of *Bifidobacteria* present. Additionally, Poroyko et al. (2010) found that *Prevotella* increased in sow fed piglets compared to formula-fed piglets in which *Bacteroides* was predominant. In this

same study, gene expression analysis revealed a significant increase in genes encoding amino acid metabolism enzymes in sow-fed piglets. Together these studies suggest a disreputable impact of milk source on the intestinal microbiota development. While these studies focus mainly on the impact of artificial vs. natural milk, cross-fostering impact should be further studied since piglets result in suckling milk from different sows (mother vs foster). It is important to study whether these sources alone, or a combination, will have a significant effect on the pig's performance and overall health.

Intestinal Bacterial Conformation and Displacement

Most studies agree *Firmicutes* and *Bacteroidetes* accounts for the majority of taxa found in fecal samples of adult humans and pigs (Guo et al., 2008; Thompson et al., 2008; Claesson et al., 2009; Park et al., 2014; Mach et al., 2015; Zhao et al., 2015) followed by *Fusobacteria*, *Proteobacteria* and *Actinobacteria*. (Zhao et al., 2015). Mach et al. (2015) described the fecal microbiota of newborn piglets to be mainly represented by *Bacteroides, Oscillibacter, Escherichia/Shigella, Lactobacillus* and unclassified *Ruminococcaceae* genera. In contrast, older pigs included *Acetivibrio, Dialister, Oribacterium, Succinivibrio* and *Prevotella* genera which is similar to Kim et al. (2011) and Looft et al. (2012) findings. Although 16S rRNA sequencing provides substantial information about bacterial communities living in a determinate location, studies suggest intestinal microbiota fluctuates between location and age (Savage, 1977; Palmer et al., 2007; Kim et al., 2011). Hence, it is important that future studies take into consideration the shifts in microbial composition across the intestine and ages. For instance, studies conducted by Thompson et al. (2008) and Zhao et al. (2015) revealed the ratio of *Firmicutes* to

Bacteroidetes from feces of older pigs was higher than that of feces of newborn pigs. Metagenomics analysis of bacterial composition in small and large intestine reveals the large intestine microbiota has higher involvement in metabolic functions compared to small intestine (Zhao et al., 2015).

Fecal sample is to some extent representative of the large intestine only and not representative of the entire GI tract (Zhao et al., 2015). Large intestine microbial species accounted for most of the species found in fecal samples and did not resemble the bacterial communities found in small intestine (Zhao et al., 2015). This novel finding needs to be taken into consideration since most of the studies so far focus on fecal microbiota and not the entire GI tract. The initial microbiota established in a piglet will shift toward a mature microbiota approximately at six months of age according to Zhao et al. (2015). Thompson et al. (2008) studied at a species level the microbial shift in 24 piglets 36 days after birth. In their findings older pigs had a more stable microbiota that resembles that of their housing littermates and not their brothers, whereas newborn pigs had a dynamic composition that resembled their brothers. Thompson et al. (2008) concluded that before the establishment of a mature and definite microbiota, there is a gap in which the pig's microbiome can be shaped and molded to produce a robust and more convenient individual. Thompson et al. (2008) also suggested further studies should focus on a species level since in their study differences could be clearly observed at a species level and not at a phyla or class level. Correspondingly Alain et al. (2014) studied the effect of diet, analyzing bacterial composition of fecal samples of pre-weaned and weaned piglets, in their results a shift in bacterial composition from *Firmicutes* to *Bacteroidetes* and an increase in *Prevotella* and *Clostridium* after weaning demonstrated that microbial communities can be influenced by diet or stress since weaning is a stressful event in the life of a pig. Likewise,

Levesque et al. (2014) found differences in mucosal bacterial composition in pigs fed with high and low energy diet, specially an increase of *Clostridium* consistent with age. Levesque et al. (2014) suggested microbial shift cannot be exclusively attributed to the diet but to the inclusion of antibiotic in the diet as well. Correspondingly, Hyeun Bum et al. (2012) revealed a prominent change at a genus level in fecal microbial composition of pigs treated with the bacteriostatic additive tylosin, a very well-known growth promoter, concluding that tylosin tends to accelerate microbial development.

In conclusion, 16S rRNA sequencing has brought up sufficient evidence to indicate that gut microbiota is fundamental to the pig's health and well-being. Moreover, that this microbiota is strongly influenced by external factors, especially the mother, a key influence upon the overall intestinal development. A healthy microbiome is key to the individual health and changes in microbial composition can lead to disease (Voreades et al., 2014). Therefore, determining a healthy microbiome in the pig's intestine and understanding its relationship with the host, especially its contribution to the immune system, can lead to the development of better management practices and more robust pigs.

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CHAPTER 2

INFLUENCE OF MATERNAL MICROBIAL COMMUNITIES ON THE MUCOSAL MICROBIOME OF NEONATAL PIGS.

INTRODUCTION

Unlike human infants and puppies, at birth piglets have exceptionally restricted body reserves and scarcely get antibodies prenatally (Decaluwé et al., 2014). They are presented to unexpected changes outside their mom's body, experiencing severe ecological difficulties transitioning from a clean uterine environment into a complex and differing microbial environment. Many internal organs including the gastrointestinal (GI) tract are still relatively immature and not prepared for extra uterine life (Sangild et al., 2013). For this reason, 80% of preweaning mortality takes place during the perinatal period, mainly during the first 3 days of life (Tuchscherer et al., 2000). This a reason for great welfare concern and conveying incredible financial misfortunes to the swine enterprise (KilBride et al., 2014). Colostrum is still the only source piglets have to receive nutrients and protection (Decaluwé et al., 2014). This significance has led to the development of different management practices to enhance the amount of colostrum received by each piglet, thus reducing piglet morbidity and mortality. Cross-fostering, the transfer of piglets between dams during the farrowing process is a necessary practice to equalize the number of piglet between litters ensuring colostrum intake for their survival and growth (Kirkden et al., 2013). While these techniques are highly effective in promoting neonatal survival, there is little known about their effects on long term piglet performance.

We know in humans microorganisms are transferred from dam to offspring at parturition and during the neonatal period (Cerf-Bensussan and Gaboriau-Routhiau, 2010). Furthermore, breast milk once considered sterile has been demonstrated to be constant sources of microbes to the newborn gut (Collado et al., 2009; Fernández et al., 2013). In humans, microbes are transmitted in a personalized manner and play a key role in the maintenance of intestinal health and homeostasis, and therefore in the prevention of diseases (Fernández et al., 2013). Recently, associations between intestinal microbiota and increased number of intestinal diseases have been described in humans (de Vos and de Vos, 2012). For instance, crohn's disease (Kaser et al., 2010; Buttót et al., 2015), celiac disease (Nistal et al., 2012; Flass et al., 2015), and increased *Clostridium* difficile infections (Grehan et al., 2010; Khoruts et al., 2010) have been associated with intestinal microbiota. In pigs, microbiota also contributes to the development of the GI microbiota influencing the immune system and playing a casual role in the incidence of diarrhea (Zhao et al., 2015). Xian et al. (2014) reported effects of cross-fostering on cecal microbiota determining differences in microbiota between fostered piglets compared to their biological siblings.

The number of studies determining the impact of cross-fostering on the GI microbiota is limited. It is our intention to increase knowledge in this area and to determine if this management practice could significantly impact the microbiota establishment during the early growing period. In view of the fact that the world is crashing into a post antibiotic era, we are in need of efficient management tools that will reduce the impact of disease without therapy and improve the nutritional needs of an increasing world population. Some important headway can be gained by taking a closer look at the interplay between the immune system, microbiota, and host. With the help of culture-independent molecular techniques we expect to have a better understanding and

assessment of the biodiversity of colostral microbiota and its relationship with the establishment and development of the gut microbiota in the growing pig. We hypothesize that cross-fostering piglets, and the timing of the cross-fostering, influences both the piglet's immune system and its microbiota, which in turn, may have an impact on lifelong performance. Hence, the aim of this study was to determine the influence of maternal microbial communities on the mucosal microbiome of the young pig subjected to cross-fostering.

MATERIALS AND METHODS

Animal Management and Experimental Design

Experimental procedures were approved by the University of Illinois Institutional Animal Care and Use Committee. Twenty-four piglets from 2 litter (12 pigs per litter), vaginally delivered from multiparous dams (White × Large) of the same parity on the same day, were enrolled in the study. Piglets were snatch farrowed at birth and placed in warm boxes under a heating lamp in the farrowing pen next to the sow. Daily physical examination including performance, appetite, and fecal score, were performed individually. Piglets were individually identified (ear tag) and stratified according gender, body weight, and good post-parturient health. Piglets were then randomly assigned to 1 of 3 treatment groups according to the source of colostrum and post-colostral milk feeding for 21 days, as follow: treatment 1 (n = 8), received colostrum and milk from their own dam; treatment 2 (n = 8), were litter exchanged at birth to receive colostrum from a foster dam for 24 – 36 hours and then returned to their own dam for post-colostral milk feeding the subsequent days; treatment 3 (n = 8), were litter exchanged at birth to receive colostrum and post-colostral milk from a foster dam, and they remained with the foster dam for the subsequent days. Each piglet was allowed to sucked colostrum for equivalent

times. The piglets were observed to exhibit vigorous teat sucking and subsequent satiation. No antibiotics were administered to the sows; *E. coli/Clostridium* bacteria vaccine was administered pre-farrowing. At birth, piglets received iron, male piglets were not castrated. Piglet's tail was not docked at this time. None of the piglets were administered antibiotics during the experimental period. All piglets were weighted directly after birth and before being euthanized.

Sample Collection

At farrowing nasal, fecal and vaginal swabs (Pur-Wraps®, Puritan Medical Products, Gulford, Maine) were collected from each sow for microbiome analysis. Sows were restrained with the use of a snare and a mouth gag in order to collect the nasal samples. Nasal and fecal swabs were collected on day 0 and 21 from each piglet for microbiome analysis, following the same procedure as in the sow.

At day 21 (a common weaning time in the pig industry), a group of 13 piglets were humanely euthanized. After opening the visceral cavity, esophagus and rectum were clamped to avoid spilling of gastrointestinal digesta and thus contamination of other intestinal parts. Immediately after removing the gastrointestinal tract from the visceral cavity, standardized locations of the stomach, ileum and mid-colon (divided into 3 equal parts) were exposed with sterile instruments and luminal contents were collected with a swab. Luminal sites were later rigorously washed several times with sterile phosphate-buffered saline (Mediatech, Inc., Manassas, VA) to remove remains of free floating bacteria and proceed to collect mucosal content. Mucosal contents from the stomach, ileum, colon, middle jejunum, distal jejunum, proximal jejunum, and duodenum were collected aseptically by scraping off the mucosa using

number 20 surgical blades (Bard-Parker, Aspen Surgical™ Products, Caledonia, MI). Mucosal scrapings were collected in cryovials and kept on dry ice until being stored at −20 °C. A 2-cm^2 portion of the tissue was excised and placed in a tube with 5 mL RNA LATER® (Sigma-Aldrich, Saint Louis, MO) for qPCR analysis. Mucosal scrapings, luminal swabs, and tissue samples were snap frozen and then stored at −20 °C. The remainder of the animals (n = 11) were penned together at weaning (day 21) and grown to market weight in pens that only contain study pigs. They were reared in a room with their farrowing cohort and cared for by farm staff according to standard practices.

DNA Isolation

Genomic DNA was extracted from 0.25 grams of mucosal scrapings (stomach, ileum, colon, duodenum, and middle, distal and proximal jejunum) and swab tips from fecal, luminal, skin and respiratory samples using the MOBIO Power Fecal DNA Isolation Kit (MO BIO Laboratories, INC., Carlsbad, CA) following the manufacturer's protocol. Samples were homogenized using the Bullet BlenderTM (Next Advance; Averill Park, NY) following the manufacturer's recommendation. Colostrum DNA was extracted using the Qiagen DNA Isolation Kit (Hilden, Germany) according to manufacture guidelines. The DNA concentration was determined with the Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop Technologies, Rockland, DE) at wavelengths of 260 and 280 nm to assess the purity of the DNA. Samples with a ratio between 1.9 and 2.15 were considered acceptable (Nanodrop Technical Note). DNA integrity was assessed by running a 2% agarose gel (Sigma-Aldrich, Saint Louis,

MO) with SYBR Safe DNA Gel Stain (Invitrogen, Grand Island, NY). Extracted DNA was stored at −20 °C.

16S rRNA Gene Amplification and Sequencing

16S rRNA genes were amplified using specific primers, F28 (5'-GAGTTTGATCNTGGCTCAG) and V1-V3 R519 (5'-GTNTTACNGCGGCKGCTG), to target the V1-V3 hypervariable region. After DNA extraction and quality assessment, 40 µl of DNA from each were place in 96 well- plate according to the sequencing laboratory directions. Samples were sent to the biotechnology center in dry ice and arrived within 30 minutes. The PCR products were sequenced using Illumina MiSeqV3 platform (Ilumina, San Diego, CA) sequencing combined with Fluidigm Access Array. Amplification technique was performed at the W. M. Keck Center for Comparative and Functional Genomics (University of Illinois, Urbana, IL). The fluidigm constructed library was quantitated by qPCR and sequenced on one MiSeq flowcell for 301 cycles from each end of the fragments using a MiSeq 600-cycle sequencing kit (version 3). Fastq files were generated and demultiplexed with the bcl2fastq v1.8.4. Conversion PhiX DNA was used as a spike-in control for MiSeq runs.

Phylogenetic Assignment and Processing of Sequenced Reads

All the total reads obtained from the sequences [230 samples; 23,870,950 reads, as follow: fecal samples generated 1,811, 829 sequences (median = 69,987; range=5,288-192,566), respiratory samples generated 1,388,305 sequences (median = 63,801; range= 4,288-156,835),

intestinal samples generated 8,816,931 sequences (median = 375,529,094; range= 15,025-183,367), and sow samples collected generated 379,752 (median = 61,399; range= 6,661-90,947) sequences], were processed together using Illinois Mayo Taxon Organization from RNA Dataset Operations (IM-Tornado; v 2.0.3.2) (Jeraldo et al., 2014) to generate Operational Taxonomical Units (OTU), and subsequently clustered into 52,642 OTUs based on 97% similarity using Greengenes as a reference database. Following sequencing, 16S rRNA gene reads were assessed for quality, only reads that were longer than cutoff lengths were processed for OTU picking. Quality scores were generated using Fast QC. All reads were initially 300 bases long, the number of bases covered by read1 and read2 were longer than the fragment length and therefore there was partial overlap between read1 and read2. To be able to run IM-TORNADO's regular pipeline, this overlap needed to be removed. For this, trimmomatic (http://www.usadellab.org/cms/?page=trimmomatic) was run to trim primer sequences at the 5' end and then cropped read1 to 250 and read2 to 200 bases long so that there will not be any overlap between read1 and read2. All the data preprocessed as described above was run with IM-TORNADO for the regionV1V3.

Diversity Indices

Alpha diversity analysis was run to know diversity within the samples or categories, while beta diversity analysis was run to determine diversity between samples or groups. The OTU table was generated by IM-TORNADO pipeline and the mapping file was required for this analysis. Quantitative insights into Microbial Ecology (QIIME; http://qiime.org/) was used to first convert Biological Observation Matrix (BIOM) file to text format file, and create a

summary of OTU table generated by IM-TORNADO to finally run alpha and beta diversity analysis scripts. Alpha diversity at several different rarefactions was calculated and then the results from all different rarefactions were collated, and then plotted as alpha rarefaction plots by QIIME. By default, the minimum rarefaction depth is 10 and the maximum is median sequence over sample count. These defaults are useful for determining whether you have enough depth in your samples to accurately capture all the diversity present. If the lines eventually plateau, then there is enough depth, otherwise the samples may not have enough depth to capture all possible OTUs present. QIIME was used to calculate beta diversity values and create a distance matrix that could be later visualized. Chao1, observed species, PD whole tree and Shannon diversity index were calculated with QIIME.

Statistical Analysis

Statistical analyses of bacterial communities were performed using JMP 12.2 (SAS Institute Inc., Cary, NC). The relative abundance of fecal bacterial taxa for each treatment group were compared using general linear models (ANOVA). The model for bacterial communities in the piglet GI tract contained the fixed effect of treatment and GI site. The model for bacterial communities in piglet nasal and fecal samples contained the fixed effects of treatment and time. The relative abundances of different bacterial taxa in each sample as covariates and sampling days as the categorical variable were used in stepwise discriminant analysis as described by Zinicola et al. (2015). In this way the microbial shift from day 1 until day 21 was illustrated using canonical loading plots. In our study, variables were removed in a stepwise manner until only variables with a $P > 0.001$ were retained in the final model. Fastq data obtained as results of

sequencing samples of sows and piglets were uploaded to the sequence read archive (SRA) on National Center for Biotechnology Information (NCBI) web page tool (http://www.ncbi.nlm.nih.gov/sra) to make the files available for a public database (BioProject ID accession number PRJNA319360). To assess the association between bacteria genera, present in colostrum, vaginal, and fecal samples of the sow with bacteria genera present in the GI tract, fecal, and nasal samples of the young pig regression and correlation analyses were performed using the REG and CORR procedures of SAS (v9.4 Institute Inc., Cary, NC). Statistical significance was declared at $P \leq 0.05$ and trends toward significance effects were noted when $0.05 < P \leq 0.10$.

RESULTS

Microbial Diversity

Reads were pooled and analyzed using various diversity metrics for each group to calculate diversity of microbial communities. The detailed diversity estimates can be found in Table 2.1. Metrics used were Chao1 index of microbial richness, observed species, and Shannon index of biodiversity. The diversity indices used represent how many different taxa were present in a sample, higher numbers indicate higher diversity. Indices of biodiversity showed higher number of microbial communities in the colon section of GI tract and in treatment 1 (Figure 2.1). Additionally, fecal and nasal samples of day 21 showed higher microbial diversity compared to samples of early piglet age (Figure 2.2).

Relative Abundance of Bacterial Phyla in the Gastrointestinal Tract of Young Pigs

The OTUs were classified into 18 bacterial phyla, of these, 5 phyla were ≥ 1%. Comparison of relative abundance at the phylum level revealed that the major phyla dominating the microbiome were *Firmicutes* 64%, *Bacteroidetes* 16%, *Proteobacteria* 12%, *Spirochaetes* 4%, and *Fusobacteria* 1% (Figure 2.3). No differences ($P > 0.05$) on the aforementioned phyla were found between treatments. However, their relative abundance was different ($P < 0.05$) among GI sites (Figure 2.3). *Firmicutes* were highly abundant through the GI tract. Greater relative abundance of *Bacteroidetes* was found in the colon compared to the other GI sites. *Proteobacteria* was observed along the GI tract with no significant differences ($P > 0.05$) between sites. Although statistical differences were not observed among GI sites, reduced relative abundance of *Proteobacteria* was observed in colon. *Spirochaetes* and *Fusobacteria* were not as predominant compared to the others abovementioned bacteria phyla. However, relative abundance of *Spirochaetes* was predominant in colon, especially in that of treatment 3.

Relative Abundance of Bacterial Phyla in Fecal Samples of Young Pigs

Relative abundance of bacterial phyla in fecal samples was not affected ($P > 0.05$) by treatment (Figure 2.4). However, relative abundance changed ($P < 0.05$) over time. The relative abundance of *Firmicutes* was predominant at day 0 but abruptly decreased at day 21. The opposite occurred with *Proteobacteria*, *Spirochaetes*, and *Bacteroidetes* which relative abundance was lower at day 0 but significantly increased at day 21.

Relative Abundance of Bacterial Phyla in Nasal Samples of Young Pigs

In nasal samples, relative abundance of bacterial phyla was not affected ($P > 0.05$) by treatment (Figure 2.5). However, relative abundance changed over time ($P < 0.05$). *Firmicutes* was highly present at day 0, but significantly decreased at day 21. The opposite occurred for *Proteobacteria,* which had lower relative abundance at day 0 but significantly increased at day 21. Similar tendency was observed for *Bacteroidetes*, although, this phylum was not as abundant as *Proteobacteria* at day 21. As we went deeper in taxonomy, we were able to see greater variation between sites, successively all samples were assessed at a genus level.

Abundance of Bacterial Genera Present in the Sows: Colostrum, Vaginal, and Fecal Samples

Bacterial communities present in colostrum, vaginal, and fecal samples collected from the sows were classified into 104 predominant bacterial genera, of which, 23 for colostrum, 21 for vaginal, and 17 for fecal were ≥ 1% (Figure 2.6). *Lactobacillus* and *Clostridium* were highly abundant in colostrum (38 and 24%, respectively) and vaginal (52 and 13%, respectively) samples. Similarly, in fecal samples *Lactobacillus* (56%), *Campylobacter* (7%), and *Anaerococcus* (5%) were the predominant genera. A large number of bacteria genera (*Allobaculum, Aminiphilus, Anaerovorax, Anoxynatronum, Barnesiella, Butyricicoccus, Butyricimonas, Cloacibacillus, Coprococcus, Corynebacterium, Escherichia/Shigella, Eubacterium, Finegold, Flavonifractor, Gilvibacter, Hydrogenobaculum, Oscillibacter, Peptoniphilus, Phascolarctobacterium, Porphyromonas, Prevotella, Pseudoflavonifractor, Pseudomonas, Ruminococcus, Saccharofermentans, Staphylococcus, Streptococcus,*

Succinivibrio, *Syntrophaceticus*, *Tannerella*, *Turicibacter*, *Veillonella*, and *Xylanibacter*) were present in less than 5% in colostrum, vaginal, and fecal samples (Figure 2.6).

Abundance of Bacterial Genera in the Gastrointestinal Tract Young Pigs

Bacterial communities present in the GI tract of young pigs were classified into 178 bacterial genera, of which, 40 were ≥ 1%. Treatment did not have a significant ($P > 0.10$) effect in bacterial communities present in the GI tract, except for *Clostridium*, *Faecalibacterium*, and *Haemophilus* (<1%) that were different ($P < 0.05$) among treatment (Table 2.2). Higher percentage of *Clostridium* and *Haemophilus* were present in the GI tract of treatment 1 and treatment 2 compared to treatment 3, whereas *Faecalibacterium* was higher in treatment 1 compared to treatment 2 and treatment 3 (Table 3). Bacterial communities were different among GI sites ($P < 0.05$), differences between GI sites and treatment are illustrated Figure 2.7. The stomach, duodenum, jejunum, ileum, and colon accounted for 17, 19, 12, 24, and 28% of total bacterial genera present in the GI tract, respectively. *Lactobacillus* and *Clostridium* were the predominant genera in stomach (51 and 11%, respectively), duodenum (61 and 65%, respectively), jejunum (56 and 24%, respectively), and ileum (15 and 34%, respectively). Colon was the most diverse section of the GI tract and no particular predominance of bacterial genera was observed (Figure 2.7). However, *Treponema* was mostly present in colon of treatment 3 (35%) compared to treatment 1 (< 1%) and treatment 2 (1%), and increased in jejunum (10%) and ileum (15%) of treatment 3. *Tannerella* was mostly present in colon (10 – 15%) and ileum (20%) in treatment 3. In a similar way, *Prevotella* was predominant in colon (15 – 20%) meanwhile in the rest of the GI tract it was present < 1%. *Ruminococcus* (8%) and *Sphaerochaeta* (5%) were mostly present in colon. Bacterial communities present in the

stomach, jejunum, and duodenum were more similar to each other than those present in ileum and colon, as represented in Figure 2.8.

Abundance of Bacterial Genera in Fecal Samples of Young Pigs

Bacterial communities present in fecal samples from piglets at day 0 and 21 were classified into 142 genera, of which, 18 were greater than 1%. Bacterial genera in fecal samples other than *Campylobacter* and *Fluviicola* were not affected ($P > 0.05$) by treatment (Table 2.3). However, abundance of these two genera was below 5%. While *Campylobacter* was mostly present in treatment 2 (5%) compared to treatment 1 (< 1%) and treatment 3 (1%), *Fluviicola* was present in treatment 1 (1%) only. Regardless of treatment, bacteria genera predominant in fecal samples were *Lactobacillus* (16%), *Clostridium* (15%), *Treponema* (12%), and *Bacteroides* (10%) with the rest accounting < 10% (Figure 2.9). Bacteria communities in fecal samples changed ($P < 0.05$) over time (Figure 2.9). While *Lactobacillus* and *Clostridium* were the predominant genera at day 0 (34 and 20%, respectively), their abundance decreased to 5% at day 21. On the other hand, *Bacteroidetes* increased from 3% at day 0 to 18% at day 21. Although other bacteria genera (*Butyricicoccus, Campylobacter, Fluviicola, and Turicibacter*) changed significantly from day 0 to day 21, their abundance was less than 5%.

Abundance of Bacterial Genera in Nasal Samples of Young Pigs

Bacterial communities present in nasal samples from piglets at day 0 and 21 were classified into 156 genera, of which, 22 were greater than 1%. Treatment did not have a significant effect ($P > 0.10$) in bacterial communities present in nasal samples, however, bacterial communities changed over time (Table 2.4). While *Clostridium* and *Lactobacillus* were

the predominant genera at day 0 (25 and 24%, respectively), their abundance completely decreased to less than 1% at day 21 (Figure 2.10). On the other hand, *Moraxella* increased from 11% at day 7 to 78% at day 21 (Figure 2.10). Although other bacteria genera (*Aerococcus, Aminiphilus, Anaerotruncus, Butyricicoccus, Clostridium, Coprococcus, Escherichia/Shigella, Ethanoligenens, Eubacterium, Facklamia, Flavonifractor, Megasphaera, Oscillibacter, Psychrobacter, Saccharofermentans,* and *Syntrophaceticus Turicibacter*) changed significantly from day 0 to day 21, their abundance was less than 5% (Figure 2.10).

Association between Bacterial Genera Present in the Sow and in the Young Pigs

Bacterial genera present in the GI tract of the piglet had the highest correlation with bacterial genera present in colostrum (r = 0.93; P <0.0001; R^2 = 0.88), vaginal (r = 0.99; P <0.0001; R^2 = 0.99), and fecal samples (r = 0.96; P <0.0001; R^2 = 0.91) of the sow (Table 2.5). Although lower than the correlations of the GI tract, bacteria genera present in fecal samples of the piglet had high correlation with bacteria genera present in colostrum (r = 0.72; P <0.0001; R^2 = 0.51), vaginal (r = 0.65; P <0.0001; R^2 = 0.41), and fecal samples (r = 0.57; P <0.0001; R^2 = 0.31) of the sow (Table 1). The lowest correlations were observed between bacteria genera present in piglets nasal samples with bacteria genera present in colostrum (r = 0.50; P <0.0001; R^2 = 0.24), vaginal (r = 0.42; P <0.0001; R^2 = 0.17), and fecal samples (r = 0.34; P <0.0001; R^2 = 0.11) of the sow (Table 2). Surprisingly, the highest correlation was between bacteria genera present in vaginal samples of the sow and the bacteria genera present in the GI tract of the young piglet.

DISCUSSION

The influence of gut microbiota in gastrointestinal diseases has successfully been demonstrated through next generation sequencing. Likewise, these techniques have revealed the importance of gut microbiota in animal gut. Clarifying normal bacterial communities versus pathogenic bacteria in the pig is pivotal for establishing differences associated with disease. In our study we used 16S rRNA sequencing to determine if cross-fostering piglets influenced the microbial communities in the developing piglet gut microbiome. The overall goal was to assess the influence of maternal microbial communities on the mucosal microbiome of the young pig subjected to cross-fostering. The current study found that the dominant phyla in the gastrointestinal tract of the young pig were *Firmicutes, Bacteroidetes,* followed by *Fusobacteria*, *Proteobacteria,* and *Actinobacteria*. These results are in accord with recent studies indicating the predominance of these particular phyla (Ley, 2008; Poroyko et al., 2010; Schmidt et al., 2011; Schokker et al., 2014). Additionally, prior studies have noted the role of microbes present in the mother responsible for colonizing the gastrointestinal tract of the young pig (Stark and Lee, 1982). Surprisingly, in our study microbial profiles of vaginal, colostrum, and feces of the sow were similar between each other with a predominance of the genera *Lactobacillus* and *Clostridium*. These genera were also found in high abundance in the gastrointestinal tract of the young pig. A possible explanation for this is that *Lactobacillus* and *Clostridium* were highly abundant in colostrum and vaginal samples of the sow and these may contribute to the colonization of the intestine of the young pig. This finding, while preliminary, suggests there is an influence of maternal colostral and vaginal microbial communities on the mucosal bacterial populations of the GI tract of the young pig. Additionally, influence of maternal microbiome seems to be highest soon after birth, but appears to diminish with time. These results are

consistent with those of Mach et al. (2015) where they reported bacterial genera being shared between sow and piglet supporting the idea of bacterial strains passed down to the offspring via breastmilk. In our study results demonstrated that the composition of fecal and nasal microbiota changed as the pigs aged. Microbial profiles of fecal and nasal samples of newborn pigs were significantly different from older pigs (21 days of age). These results are consistent with those of Thompson et al. (2008) who agreed that bacterial ratio changes with age and more specifically that *Bacteroidetes* in feces increased with age. This matches our results in which we found an increase in *Bacteroidetes* at day 21.

Our results suggest that microbes vary throughout the GI tract, these results are in accord with Isaacson and Kim (2012), microbial communities found in the small intestine (jejunum, duodenum, ileum) were different than that found in the large intestine (colon). Colon was very diverse and no predominance of genera was observed, however, *Treponema* was most abundant in colon specifically in treatment 3. *Treponema* are fastidious and difficult to cultivate, it may be found in vaginal, oral, and GI tract of humans, animals and insects. Recent identification of *Treponema* relies solely on metagenomics techniques (Evans et al., 2011). The finding of *Treponema* in our study can provide some insight into commensal and pathogenic *Treponemas*, although further phylogenetic studies are necessary to elucidate this, and be used as biomarkers for future diseases. According to DiBaise et al. (2008) these locations have different metabolic functions and therefore the microbes vary between locations. For instance, the small intestine is in charge of digestibility and absorption, meanwhile large intestine contains large number of bacteria in charge of fermentation. Furthermore, changes in gut microbiota have been associated with caloric intake and body weight (Park et al., 2014) as well as host genetics (Ley, 2008; Benson et al., 2010). In our study, we did not take into consideration body weight or genetics; it

is possible this may contribute to the changes in microbial communities. In contrast to previous findings (Zhao et al., 2015), *Firmicutes* was more abundant in the small intestine and *Bacteroidetes* was increased in the large intestine. Fecal samples showed higher abundance of *Bacteroidetes* at day 21. It is possible therefore, that feces were mainly representative of the large intestine and not representative of the entire GI tract (Zhao et al., 2015). *Proteobacteria* and *Actinobacteria* were present mainly in nasal samples. In the GI tract, treatment had an effect on *Clostridium*, *Faecalibacterium*, and *Haemophilus*, it is possible therefore, that cross-fostering did impact the trajectory of certain genera in the development of the mucosal microbiome. Although not statistically different some genera were present on treatment 3 that were not present on treatment 1 or treatment 2 such is the case of *Verrucomicrobia*. According to Dubourg et al. (2013), the prevalence of this particular phyla and *Synergistetes* may suggest dysbiosis and a risk to the health of the pig's gut. In our study *Synergistetes* remained steady especially in the colon. Vianna et al. (2007) found that although this phylum is normal microbiota, its high abundance could potentially play a role in periodontal disease. Although in this study we could not determine a difference and/or association between treatments and these two phyla, the prevalence and quantity of these bacteria may be suggestive of a dysbiotic gut.

CONCLUSION

This project is a unique assessment of microbial populations within the context of cross fostering and efforts were made to address possible confounding factors and maintain conclusions within limitations of the experimental design. Overall the results from this study revealed the predominance of the phylum *Firmicutes* and *Bacteroidetes* in the gastrointestinal

tract of the young pig, the gastrointestinal tract of the young pig is highly diverse specially the colon. Most of the variability was noticed at a genus level, meaning that bacteria present in a low quantity could be key microbes to understand the functions of the microbiome and its relationship with disease. There were a low number of pathogenic species (<1%) that could potentially cause disease, this needs to be further investigated. Furthermore, there is an influence of maternal microbial populations on the development of the newborn pig and this microbiota continues to change as the pig grows. Changes in microbial communities although not addressed in this study may be caused by a variety of factors, including environment, antibiotic, stress and genetics. The vast majority of gut microbiota studies have focused on the descriptions of the bacteria present in the gut. However, future trials should assess the dynamics of gut microbiota, its translation to function and the effect of these functions on health and well-being. This will likely provide researchers with crucial information that will be used to improve productivity in food animals. The microorganisms present in different GI sites resemble those present in colostrum and vaginal. This allows us to hypothesize a strong influence of colostrum and vaginal in the development of the gastrointestinal microbiota of the newborn piglet. Techniques such as metatranscriptomics and metabolomics will be needed to reveal causes and effects of microbial shifts in the gut and could potentially reveal biomarkers of disease before clinical symptoms appear.

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Table 2.1. Microbial diversity estimates

Piglet GI[1] site	Shannon[2]	SD[3]	Chao[4]	SD	Observed sp[5]	SD
Piglet GI site						
Stomach	5.33	0.92	88.0	1.39	86.0	5.87
Duodenum	4.86	0.97	66.3	6.38	74.7	8.81
Jejunum	5.26	0.63	86.9	1.32	68.8	2.37
Ileum	5.31	0.86	100.6	2.23	75.0	3.22
Colon	6.90	0.65	106.5	4.79	135.7	9.96
Piglet						
Fecal	3.76	0.32	100.1	1.25	74.4	3.96
Nasal	4.19	1.52	103.6	0.25	135.6	9.70
Piglet fecal by day						
0	3.78	0.56	65.3	0.52	66.0	7.56
21	4.26	0.76	105.6	3.56	139.0	9.57
Piglet nasal by day						
0	3.13	0.57	28.0	5.23	46.8	8.56
21	4.36	0.60	32.6	6.24	105.6	3.26
Sow						
Colostrum	4.63	0.30	103.7	8.90	120.1	5.20
Fecal	3.79	0.68	172.2	9.60	120.8	10.26
Vaginal	4.31	0.24	126.5	3.23	147.7	8.39
Overall						
Sow	4.05	0.62	157.6	4.56	137.6	8.45
Treatment 1	4.02	1.12	106.8	5.11	81.7	4.65
Treatment 2	3.86	1.12	96.2	5.23	76.5	0.57
Treatment 3	3.86	0.88	92.7	4.56	67.2	1.24
Overall day						
0	4.23	0.50	121.3	1.24	86.2	0.32
21	5.79	1.08	95.2	2.45	173.3	3.78

[1] Gastrointestinal.
[2] Shannon index of biodiversity.
[3] Standard deviation.
[4] Chao 1 index of microbial richness.
[5] Observed species.

Table 2.2. Bacteria genera present in the different gastrointestinal (GI) tract of piglets in treatment 1, 2, and 3

Phylum	Genus	Treatment[1]			SEM[2]	GI Site					SEM	*P*[3]	
		1	2	3		Colon	Duodenum	Ileum	Jejunum	Stomach		Trt	GI site
Bacteroidetes	Alloprevotella	1.31	1.83	0.59	0.34	1.74	0.79	0.31	0.68	3.02	0.52	0.09	0.02
Firmicutes	Anaerostipes	0.16	0.18	0.03	0.06	0.44	0.10	0.07	0.06	0.14	0.09	0.19	0.03
Bacteriodetes	Bacteroides	2.94	1.37	1.74	0.74	5.58	1.86	4.34	1.68	1.22	1.13	0.35	0.05
Bacteroidetes	Bergeyella	1.03	0.80	0.50	0.18	0.25	1.57	0.26	0.61	2.45	0.28	0.19	0.001
Firmicutes	Clostridium	16.2	21.0	4.40	4.05	6.72	7.94	29.1	24.8	12.5	6.18	0.05	0.05
Firmicutes	Dorea	0.36	0.24	0.58	0.17	1.24	0.24	-0.01	0.12	0.21	0.25	0.38	0.02
Firmicutes	Faecalibacterium	0.55	0.09	0.01	0.12	0.67	0.55	0.70	0.39	0.43	0.19	0.03	0.60
Proteobacteria	Haemophilus	0.14	0.26	0.05	0.06	0.02	0.05	0.21	0.09	0.31	0.09	0.05	0.12
Bacteriodetes	Hallella	0.13	0.14	0.14	0.04	0.49	0.00	0.12	0.05	0.01	0.06	0.98	0.001
Firmicutes	Lactobacillus	37.1	32.3	43.4	4.96	4.00	61.9	14.5	51.8	53.4	7.58	0.36	0.001
Proteobacteria	Moraxella	3.19	1.43	2.30	0.75	0.89	5.24	0.89	2.67	6.28	1.15	0.31	0.01
Proteobacteria	Paraperlucidibaca	0.28	0.09	0.10	0.07	0.13	0.24	0.13	0.34	0.58	0.11	0.15	0.04
Bacteriodetes	Porphyromonas	1.04	0.90	1.05	0.20	0.04	1.06	0.04	0.76	3.30	0.31	0.85	0.001
Bacteroidetes	Prevotella	2.24	2.0	2.55	0.63	9.58	0.36	0.44	0.27	0.54	0.97	0.83	0.001
Firmicutes	Ruminococcus	1.54	1.76	2.47	0.73	5.39	0.97	0.83	0.43	0.05	1.11	0.65	0.02
Spirochaetes	Treponema	1.45	0.67	2.02	0.40	5.65	0.14	0.92	0.26	0.26	0.61	0.11	0.0002
Fusobacteria	Streptobacillus	0.07	0.11	0.11	0.03	-0.03	-0.01	-0.03	0.02	0.40	0.05	0.59	0.003
Firmicutes	Streptococcus	1.01	0.99	0.60	0.23	0.17	2.35	0.33	0.85	1.37	0.35	0.40	0.01
Firmicutes	Turicibacter	0.66	0.92	0.61	0.21	0.08	0.60	0.63	0.36	1.61	0.32	0.54	0.03

[1] Treatment: 1 = litter received colostrum and post-colostrum milk feeding from their own dam; 2 = litter exchanged at birth to receive colostrum from a foster dam and then returned to their own dam for post-colostral milk feeding for the subsequent days; and 3 = litter exchanged at birth to receive colostrum and post-colostrum milk feeding from a foster dam, they remained with the foster dam for the subsequent days.[2] Greatest standard error of mean (SEM).[3] Trt = treatment; GI site = gastrointestinal site

Table 2.3. Bacteria genera present in fecal samples of treatment 1, 2, and 3 at day 0 and 21

Phyla	Genera	Treatment[1]			SEM[2]	Day		SEM	*P*[3]	
		1	2	3		0	21		Trt	Day
Bacteroidetes	Bacteroides	2.73	5.10	-2.09	5.09	2.73	17.91	5.09	0.40	0.09
Firmicutes	Butyricicoccus	1.53	1.03	1.00	0.33	1.53	0.35	0.33	0.40	0.06
Proteobacteria	Campylobacter	-0.39	4.56	-0.02	0.47	-0.39	1.31	0.47	0.02	0.06
Firmicutes	Clostridium	19.99	26.46	19.78	1.96	20.0	4.78	1.96	0.15	0.01
Bacteroidetes	Fluviicola	0.46	-0.02	-0.05	0.07	0.46	0.56	0.07	0.04	0.20
Firmicutes	Lactobacillus	34	24	37	5.09	34.3	5.03	5.09	0.30	0.02
Firmicutes	Turicibacter	5.08	5.79	6.44	0.74	5.08	-0.37	0.74	0.46	0.01

[1] Treatment: 1 = litter received colostrum and post-colostrum milk feeding from their own dam; 2 = litter exchanged at birth to receive colostrum from a foster dam and then returned to their own dam for post-colostral milk feeding for the subsequent days; and 3 = litter exchanged at birth to receive colostrum and post-colostrum milk feeding from a foster dam, they remained with the foster dam for the subsequent days.
[2] Greatest standard error of mean (SEM).
[3] Trt = treatment; Day = 0 and 21.

Table 2.4. Bacteria genera present in nasal samples of treatment 1, 2, and 3 at day 0 and 21

Phylum	Genera	Treatment			SEM	Day		SEM	*P*	
		1	2	3		0	21		Trt	Day
Firmicutes	Aerococcus	1.15	0.98	0.85	0.15	1.15	0.17	0.15	0.43	0.02
Synergistetes	Aminiphilus	0.28	0.41	0.38	0.07	0.03	0.01	0.08	0.44	0.04
Firmicutes	Anaerotruncus	0.41	0.45	0.37	0.05	0.41	0.00	0.05	0.50	0.01
Firmicutes	Butyricicoccus	0.24	0.25	0.25	0.01	0.24	-0.01	0.01	0.50	0.01
Firmicutes	Clostridium	25.1	25.2	27.0	2.23	25.05	0.66	2.23	0.76	0.01
Firmicutes	Coprococcus	2.76	3.26	3.91	0.73	2.76	-0.42	0.73	0.54	0.04
Proteobacteria	Escherichia/Shigella	1.08	0.74	1.03	0.21	1.08	0.13	0.21	0.50	0.04
Firmicutes	Ethanoligenens	0.03	0.03	0.03	0.00	0.03	0.00	0.00	0.50	0.01
Firmicutes	Eubacterium	0.34	0.28	0.35	0.05	0.34	0.03	0.05	0.62	0.02
Firmicutes	Facklamia	0.28	0.06	0.39	0.22	0.28	1.84	0.22	0.55	0.01
Firmicutes	Flavonifractor	0.39	0.37	0.48	0.06	0.39	-0.02	0.06	0.50	0.02
Firmicutes	Lactobacillus	23.8	25.3	25.7	1.07	23.8	-0.8	1.07	0.48	0.01
Firmicutes	Megasphaera	1.10	1.48	1.10	0.25	1.10	-0.12	0.25	0.50	0.04
Proteobacteria	Moraxella	10.94	2.14	9.66	9.07	10.9	78.0	9.07	0.73	0.02
Firmicutes	Oscillibacter	0.77	0.99	0.92	0.11	0.77	-0.04	0.11	0.40	0.01
Firmicutes	Psychrobacter	1.20	1.23	1.19	0.03	1.20	-0.002	0.03	0.63	0.01
Firmicutes	Saccharofermentans	0.57	0.71	0.74	0.11	0.57	-0.09	0.11	0.50	0.03
Firmicutes	Syntrophaceticus	0.65	0.78	0.86	0.14	0.65	-0.06	0.14	0.58	0.03
Firmicutes	Turicibacter	3.33	3.18	2.44	0.51	3.33	0.52	0.51	0.46	0.03

[1] Treatment: 1 = litter received colostrum and post-colostrum milk feeding from their own dam; 2 = litter exchanged at birth to receive colostrum from a foster dam and then returned to their own dam for post-colostral milk feeding for the subsequent days; and 3 = litter exchanged at birth to receive colostrum and post-colostrum milk feeding from a foster dam, they remained with the foster dam for the subsequent days.

[2] Greatest standard error of mean (SEM).

[3] Trt = treatment; Day = 0 and 21.

Table 2.5 Association between bacteria genera present in colostrum, vaginal, and fecal samples of the sow and bacteria genera present in the gastrointestinal (GI) tract, fecal, and nasal samples of the young piglet

	Piglet			
Sow	GI Tract	Fecal	Nasal	
Colostrum	0.93806	0.71836	0.49668	r
	<0.0001	<0.0001	<0.0001	*P*
	0.88	0.51	0.24	R^2
Vaginal	0.99982	0.64664	0.42366	r
	<0.0001	<0.0001	<0.0001	*P*
	0.99	0.41	0.17	R^2
Fecal	0.95626	0.56564	0.34129	r
	<0.0001	<0.0001	<0.0001	*P*
	0.91	0.31	0.11	R^2

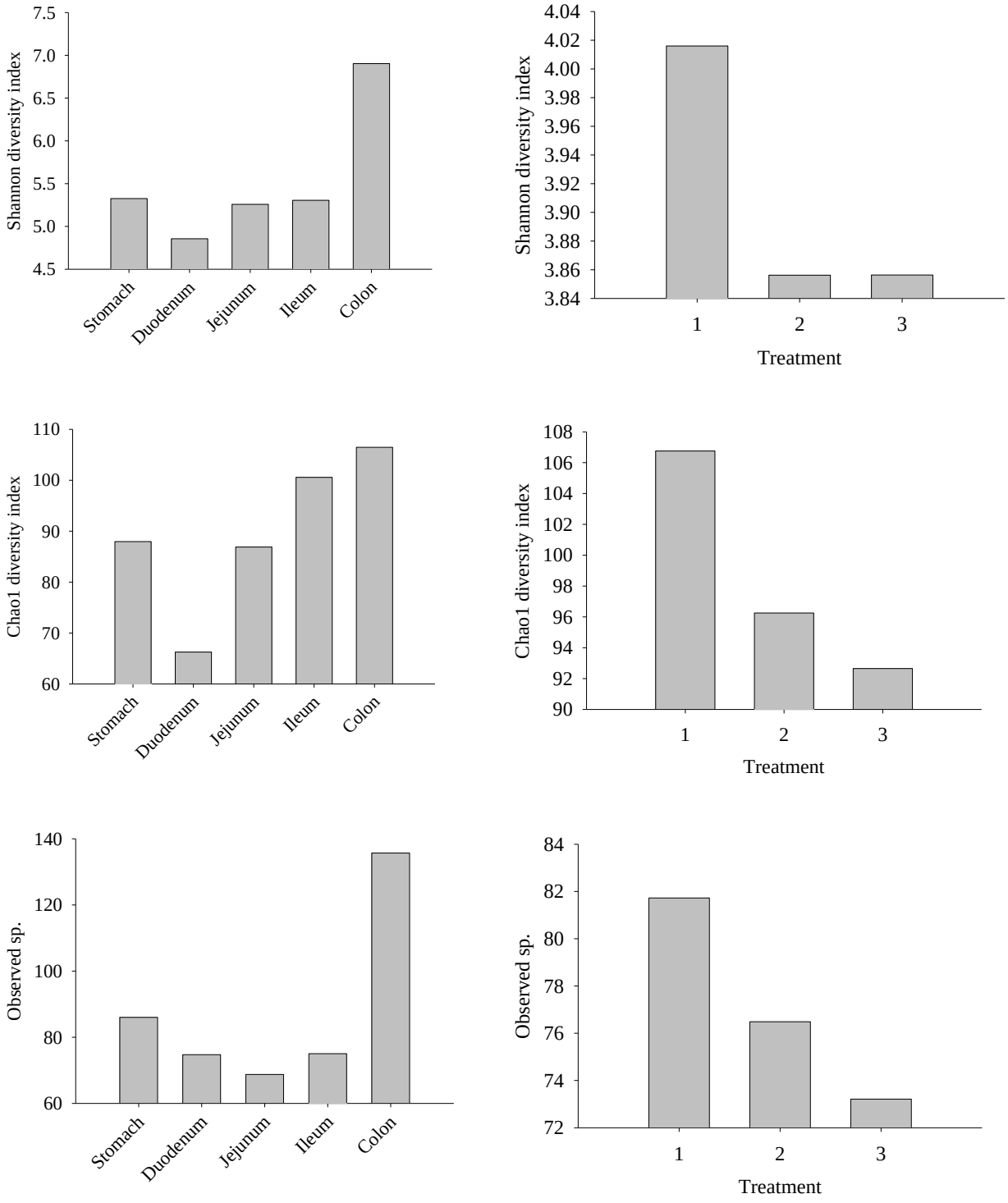

Figure 2.1. Microbial diversity estimates by gastrointestinal site and treatment.

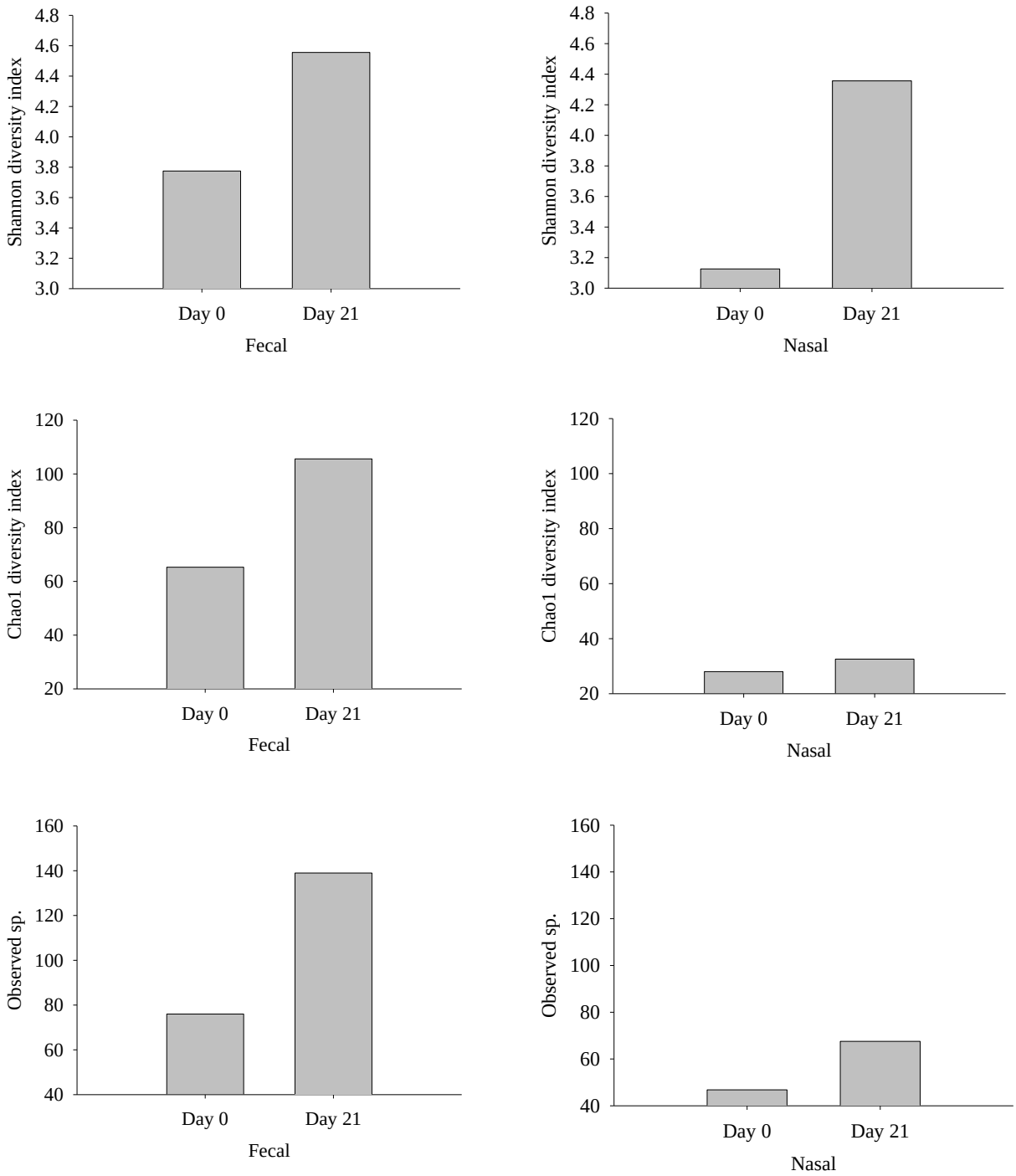

Figure 2.2. Microbial diversity estimates in fecal and nasal samples at day 0 and 21.

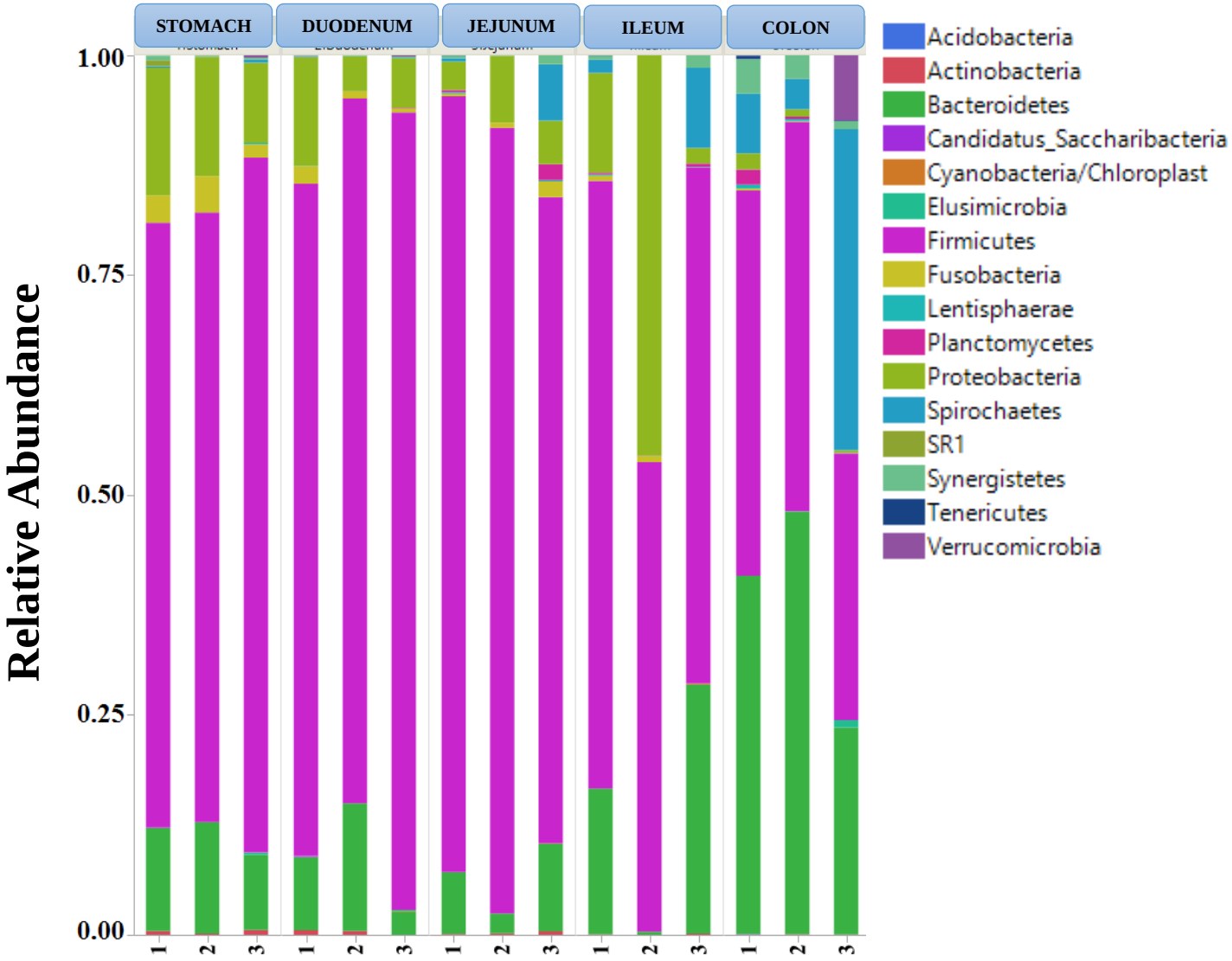

Figure 2.3. Bacterial phyla present in the gastrointestinal tract of the young pig by treatment.

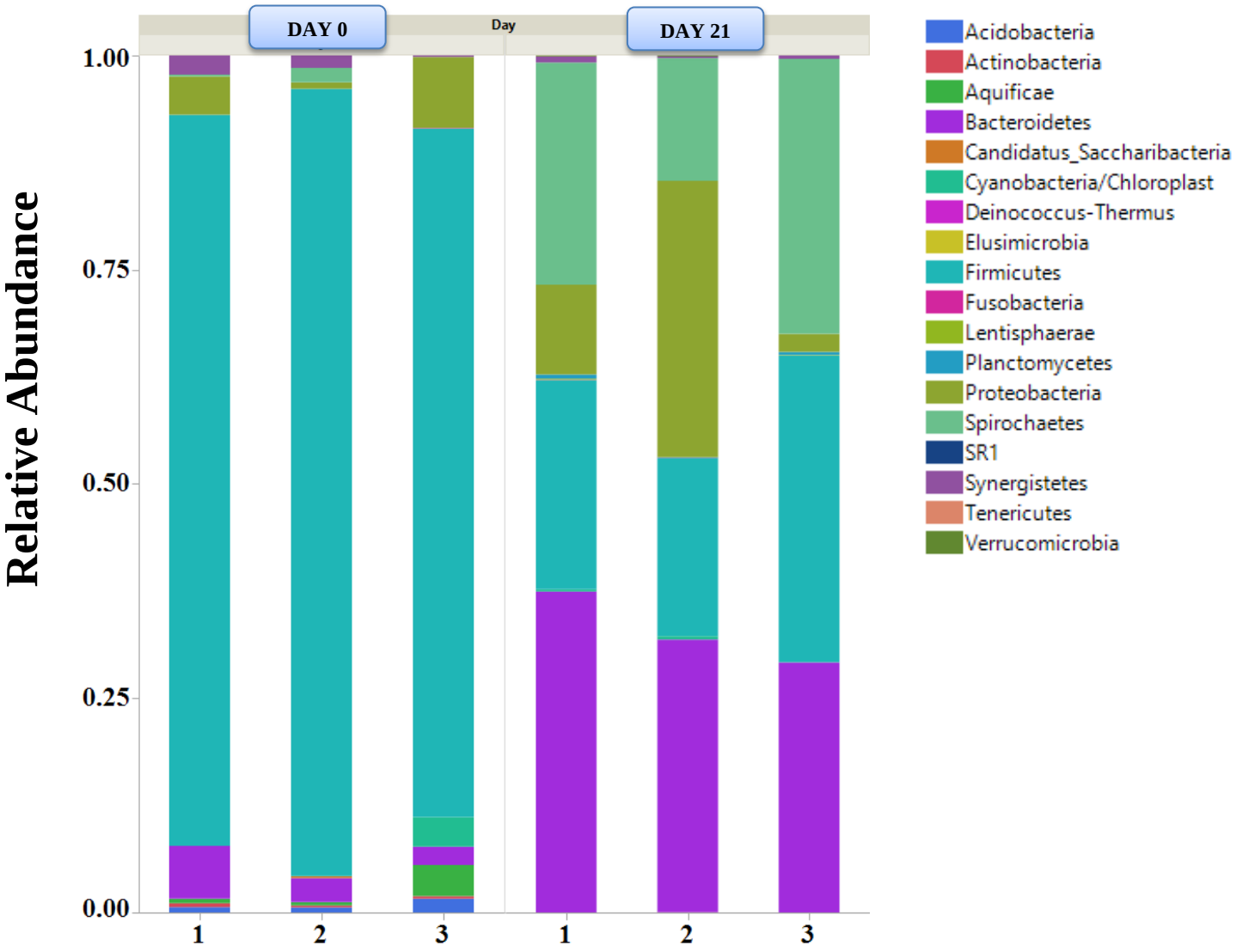

Figure 2.4. Bacterial phyla present in fecal samples at day 0 and 21 of the young pig by treatment.

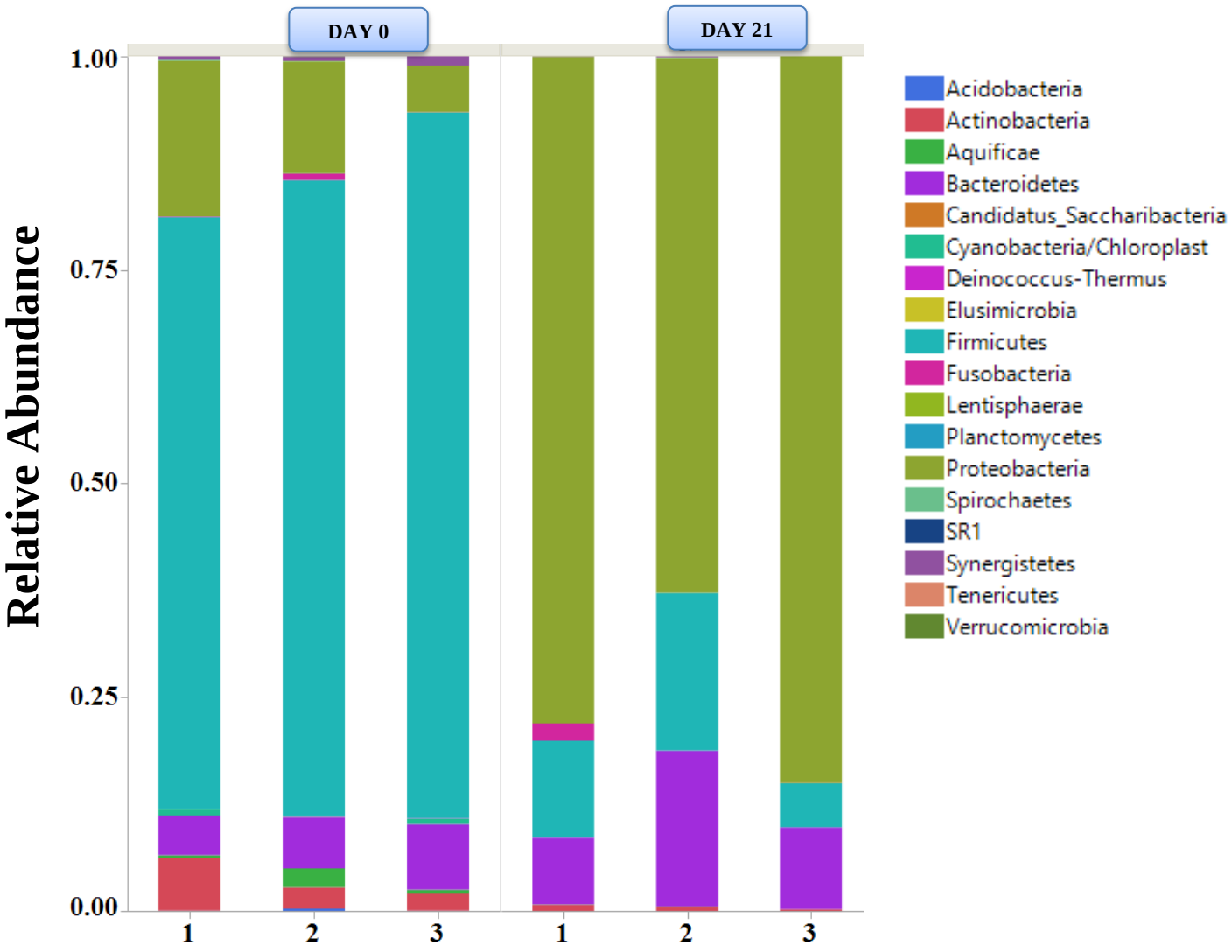

Figure 2.5. Bacterial phyla present in nasal samples at day 0 and 21 of the young pig by treatment.

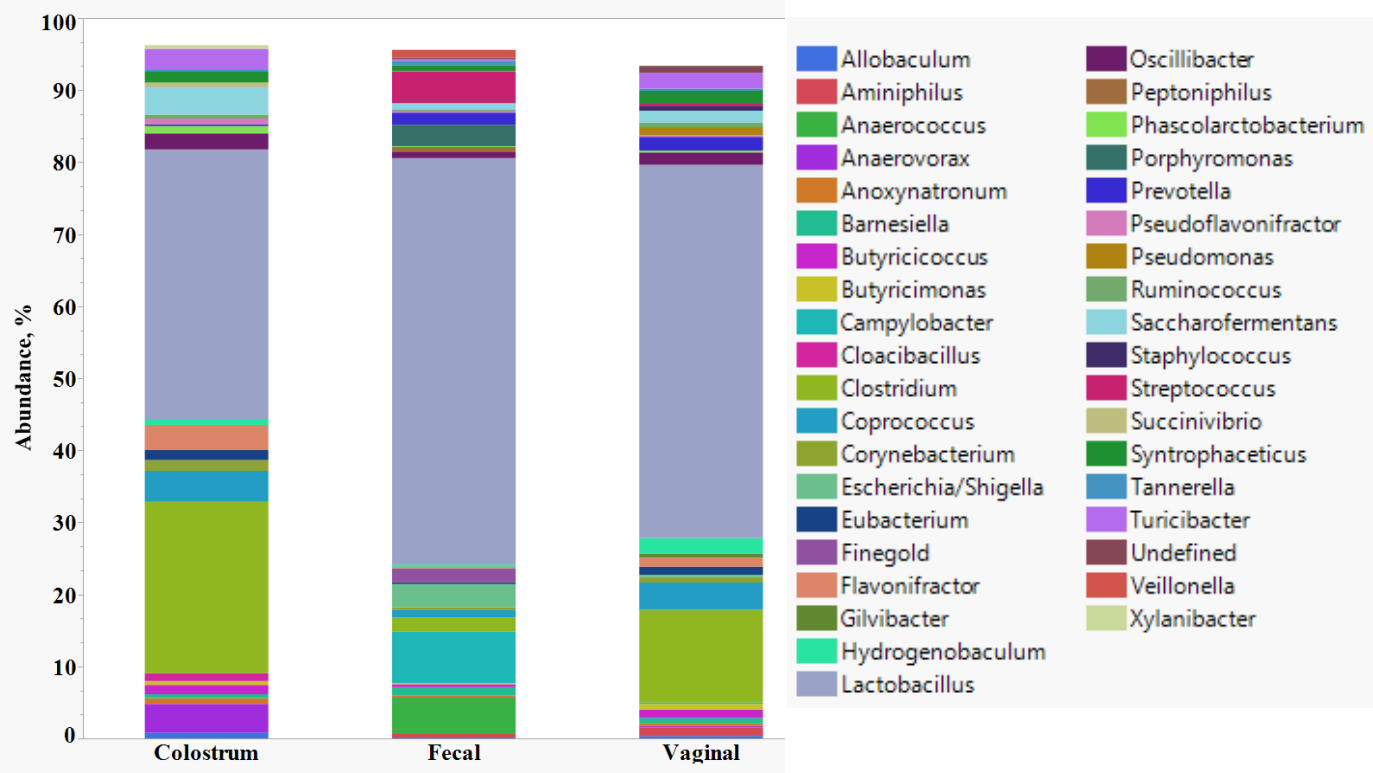

Figure 2.6. Bacterial genera present in colostral, fecal, and vaginal of the sow.

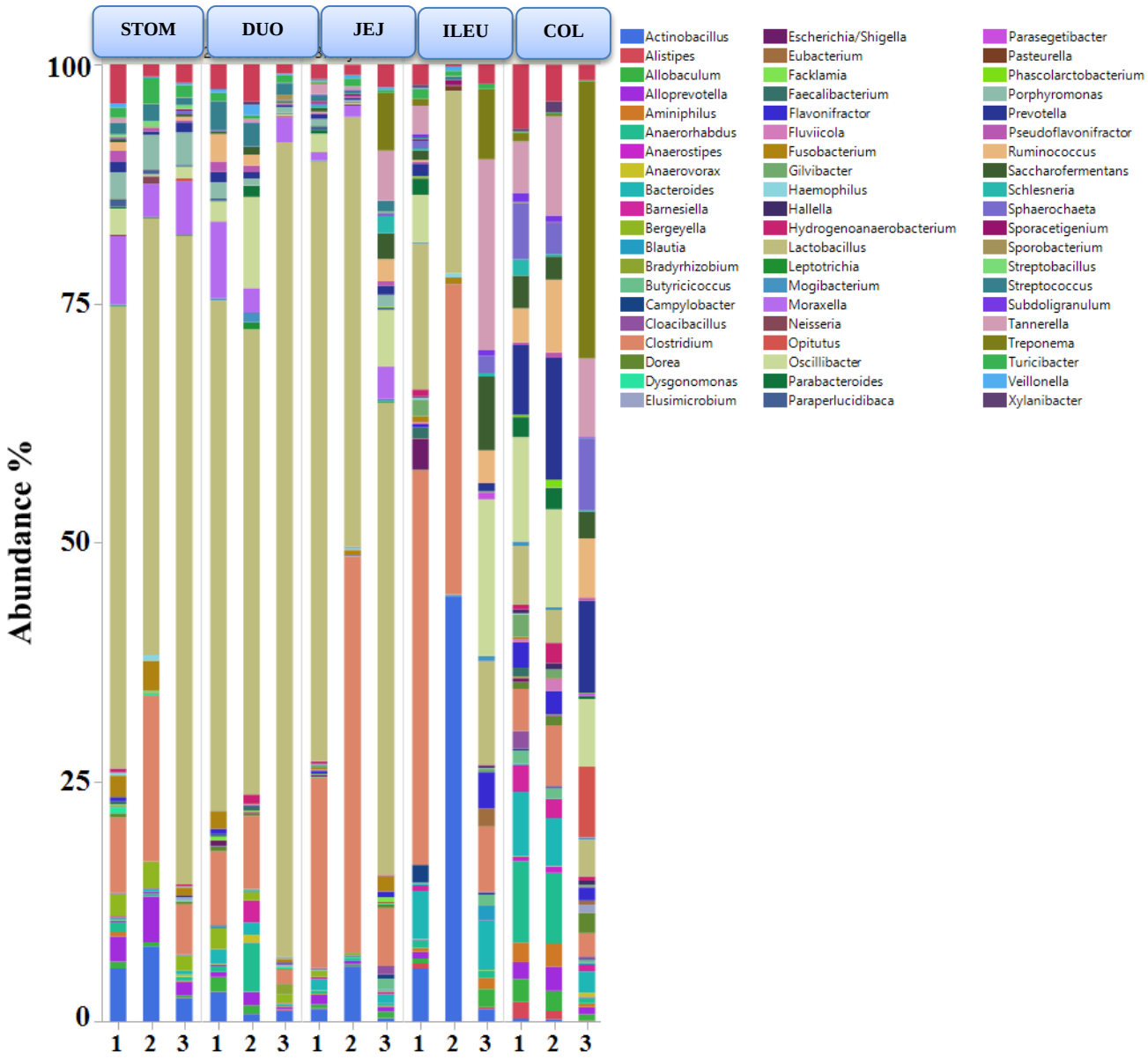

Figure 2.7. Bacterial genera present in the gastrointestinal tract of the young pig by treatment.

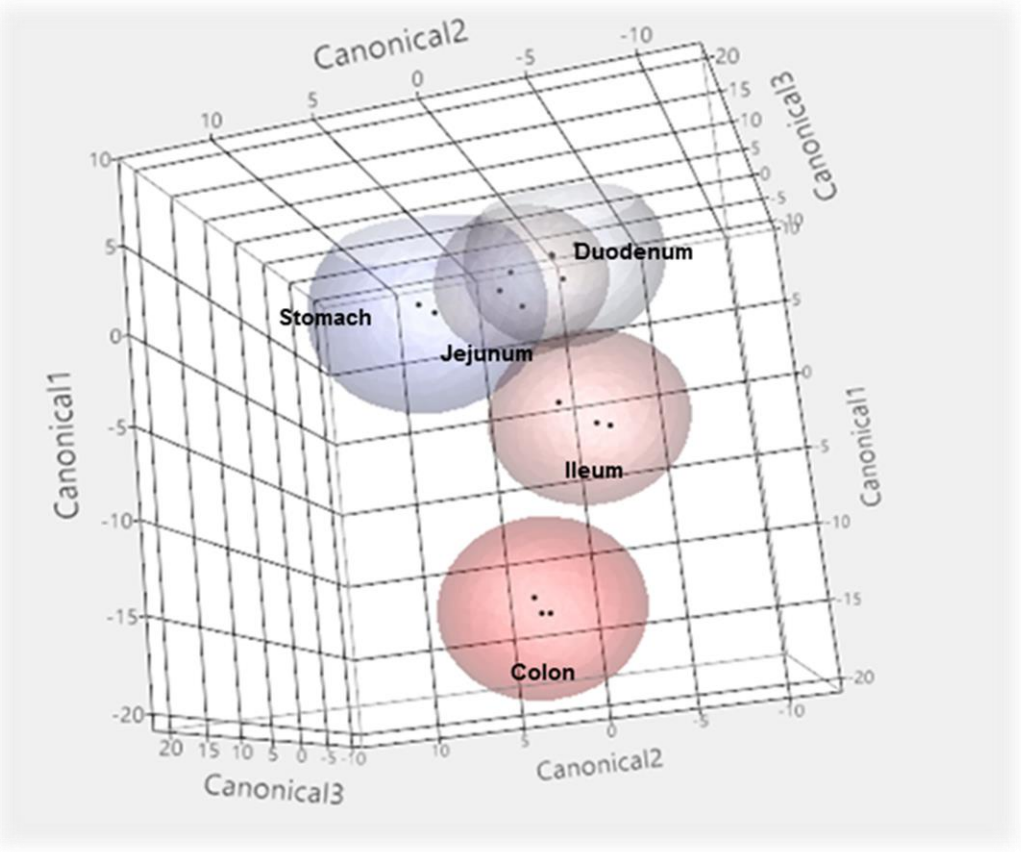

Figure 2.8. Discriminant analysis of bacterial genera present in the gastrointestinal tract of the young pig.

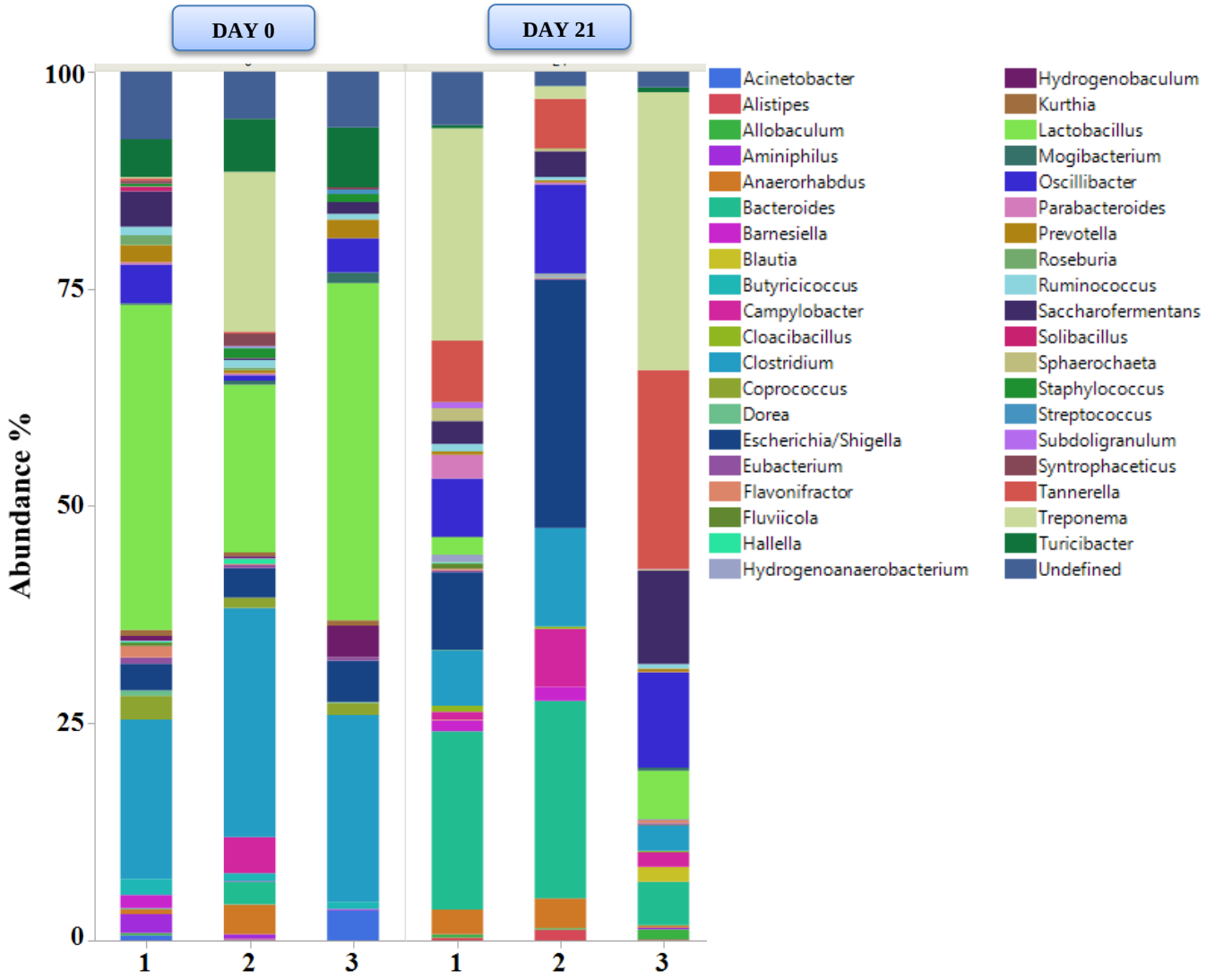

Figure 2.9. Bacterial genera present in fecal samples of the young pig by day and treatment

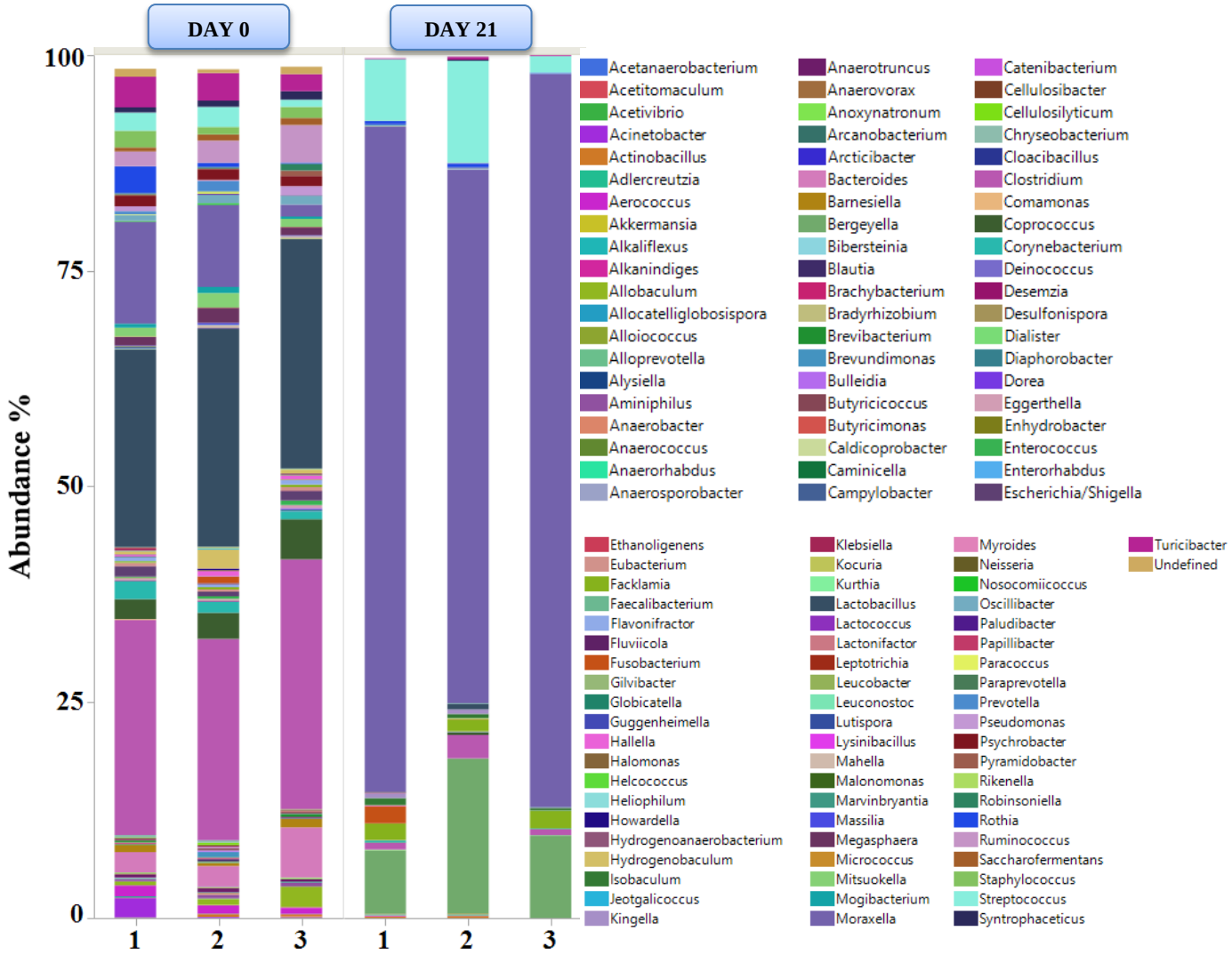

Figure 2.10. Bacterial genera present in nasal samples of the young pig by day and treatment

CHAPTER 3

EXPRESSION OF TOLL-LIKE RECEPTORS AND INFLAMMATORY CYTOKINES IN GUT-ASSOCIATED LYMPHOID TISSUES IN NEONATAL PIGS.

INTRODUCTION

Gastrointestinal disease (GI) is the leading cause of morbidity and economic loss in the swine industry (Phillips et al., 2014). Lactation remains a crucial phase in the swine industry for piglet survival, for this reason, several management strategies such as colostrum or cross-fostering have been implemented to ensure piglet survival and growth (Muns et al., 2013). Soon after birth the pig mucosal barrier plays an important role in the pig's health and protection, and most of this protection is conferred by the mother immediately after birth through the administration of colostrum (Levast et al., 2014). Initially, piglets are born immunocompetent; an impermeable placenta prevents the circulatory transfer of immunoglobulins from pregnant sows to piglet. Therefore, newborn pigs must obtain immune protection through colostrum and post colostral milk; immunoglobulins from the mother are transferred in milk until weaning. Secretory IgA (sIgA) which is responsible of protecting the intestine of the developing pig (Evans et al., 1980) is transferred through colostrum and milk. For this reason, the newborn pig must consume colostrum during the first 3 days of life to receive a proper initial immune protection (Salmon et al., 2009; Levast et al., 2014). The recruitment of sIgA secreting plasma cells and maturation of gut associated lymphoid tissues (GALT) is initiated soon after birth and reaches maturation until 5 weeks of age (Levast et al., 2014), both plasma cells and GALT are stimulated by the initial microbiota. Studies have demonstrated the sole presence of bacteria

accounts for more than 70% of the sIgA secreted in the intestine (Macpherson and Uhr, 2004). Starting at birth newborns pigs are exposed to microbes in the environment and in the milk (Salmon et al., 2009). Several studies have demonstrated a relationship between antigenic stimulation within the intestine by these microbes and antibody production (Evans et al., 1980). Thereby, a homeostatic relationship can be described between the immune system and the microbiota residing in the intestine in which equilibrium is established through positive and negative feedback (Hooper and Macpherson, 2010). Microbial–associated molecular patterns (MAMPs) are conserved and necessary structures for pathogens, these structures elicit an immune response when recognized by epithelial cells through pattern recognition receptors (PRR) (Newman et al., 2013). The host activates several signaling cascades that lead to the production of chemokines and antimicrobial peptides (AMPSs), crucial elements in the protection against gastrointestinal infections (Cerf-Bensussan and Gaboriau-Routhiau, 2010; Cederlund et al., 2013). The toll-like receptors (TLR) are a type of PRR expressed on epithelial and immune cells that recognize specific bacterial structures such as lipopolysaccharides and promote signaling and inflammation (Balachandran et al., 2015). Together TLR and other binding proteins result in a translocation of transcription factor termed nuclear factor kappa-light-chain-enhancer of activated β cells (NF-KB) which in result activates proinflammatory cytokines. Therefore, the establishment of this initial microbiota helps stimulate the immune system development in the newborn pig (Suzuki and Fagarasan, 2008). As part of a study assessing microbial populations in newborn pigs, this study sought to establish a baseline for mucosal immune gene expression in young pigs reared in a cross-fostering model given high quality colostrum from birth dam or foster dam upon birth.

MATERIALS AND METHODS

Animal Management and Experimental Design

Experimental procedures were approved by the University of Illinois Institutional Animal Care and Use Committee. Twenty-four piglets from 2 litter (12 pigs per litter), vaginally delivered from multiparous dams (White × Large) of the same parity on the same day, were enrolled in the study. Piglets were snatch farrowed at birth and placed in warm boxes under a heating lamp in the farrowing pen next to the sow. Daily physical examination including performance, appetite, and fecal score, were performed individually. Piglets were individually identified (ear tag) and stratified according gender, body weight, and good post-parturient health. Piglets were then randomly assigned to 1 of 3 treatment groups according to the source of colostrum and post-colostral milk feeding for 21 days, as follow: treatment 1 (n = 8), received colostrum and milk from their own dam; treatment 2 (n = 8), were litter exchanged at birth to receive colostrum from a foster dam for 24 – 36 hours and then returned to their own dam for post-colostral milk feeding the subsequent days; treatment 3 (n = 8), were litter exchanged at birth to receive colostrum and post-colostral milk from a foster dam, and they remained with the foster dam for the subsequent days. Each piglet was allowed to suckle colostrum for equivalent times. The piglets were observed to exhibit vigorous teat sucking and subsequent satiation. No antibiotics were administered to the sows; *E. coli*/*Clostridium* bacteria vaccine was administered pre-farrowing. At birth, piglets received iron, male piglets were not castrated. None of the piglets were administered antibiotics during the experimental period. All piglets were weight directly after birth and before being euthanized.

Sample Collection

A group of 13 piglets were humanely euthanized at day 21 (a common weaning time in the pig industry). After opening the visceral cavity, esophagus and rectum were clamped to avoid spilling of gastrointestinal digesta and thus contamination of other intestinal parts. Immediately after removing the gastrointestinal tract from the visceral cavity, standardized locations of the stomach, jejunum, ileum, and colon were exposed with sterile instruments. A 2 cm^2 portion of the tissue was excised and placed in a tube with 5 mL RNA LATER® (Sigma-Aldrich, Saint Louis, MO) for quantitative real-time PCR (qPCR) analysis and stored at -80 upon arrival. The remainder of the animals (n = 11) were penned together at weaning (day 21) and grown to market weight in pens that only contain study pigs. They were reared in a room with their farrowing cohort and cared for by farm staff according to standard practices.

RNA Isolation, Quality Assessment, and cDNA Synthesis

Intestinal samples from jejunum, jejunum peyer's patches, jejunum lymph node, ileum, ileum lymph node, ileum peyer's patches, colon, and colon lymph node were independently processed. Two cm^2 of each intestinal tissue were cut and stored in RNA LATER® according to manufacturer's instructions. Extraction of RNA was achieved using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) and following manufacturer's directions for animal tissue. Thirty mg of intestinal mucosa were isolated by cutting with a sterile surgical scalpel blade and homogenizing in 600 µL of RLT buffer (Qiagen, Valencia, CA) using the Bullet BlenderTM (Next Advance) in which dry ice could be incorporated during the homogenization of samples. Less than 30 mg of tissue was recommended for tougher tissues such as lymph nodes. Samples were stored at −80

°C at all times. The RNA concentration was measured with Nanodrop ND-1000 spectrophotometer. The purity of RNA was assessed by ratio of optical density OD 260/280, which was above 1.9 for all samples. Following RNA isolation, a portion of the RNA was diluted with DNase/Rnase free water (Sigma-Aldrich, Saint Louis, MO) for cDNA synthesis through reversed transcriptase PCR. The cDNA was synthesized using 100 ng of RNA and diluted with nuclease free water. The mix was prepared by combining 80 µL of diluted RNA with 20 µL of qScript DNA SuperMix (Quanta Biosciences, Beverly, MA). Reaction mixtures were incubated in a BioRad T100 Thermal Cycler for 5 minutes at 25 °C, 30 minutes at 42 °C, 5 minutes at 85 °C, and held at 4 °C indefinitely. The cDNA was then diluted 1:4 with DNase/Rnase free water.

Primer Design and Evaluation

Forward and reverse primers for TLR 2, 4, and 10, tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), interleukin 2 (IL2), interleukin 4 (IL4), and the house keeping genes beta 2 microglobulin (β2M), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and beta-actin (ACTB) were obtained from Integrated DNA Technologies (IDT, Coralville, IA). Table 3.1 shows the list of all primers used for qPCR analysis in this study. Then, primers were aligned against NCBI database through BLASTN (blast.ncbi.nlm.nih.gov) to determine the compatibility of primers with already annotated sequence of the corresponding gene in the database. Primers were reconstituted with nuclease free water in the amount of µL equal to 10 times the number of nMoles. Prior to qPCR, primers were verified through a 25 µL PCR reaction, which followed the same procedures for qPCR. Four µL of PCR product was run in a 2% agarose gel stained with SYBR Safe Gel Stain (Invitrogen, Carlsbad, CA), and the remaining was cleaned with QIAquick

PCR Purification Kit and sequenced at the Core DNA sequencing facility at the University of Illinois. The sequencing product was confirmed through BLASTN at the National Center for Biotechnology Information (NCBI) database. Only primers that presented a single band of the expected size and the right amplification product were used for qPCR.

Quantitative Real-Time PCR

Amplification and qPCR measurements were performed using the Applied Biosystems 7500® Real-Time PCR with version 2.0.6 software. Experiments were performed in 96-well plates. Within in each well, 10 µL of diluted cDNA combined with 12.5 µL of Perfecta SYBR Green (Thermo Fisher Scientific Waltham, MA), 0.1 µL of forward primer, 0.1 µL of reverse primer, and 2.3 µL of nuclease free water. Five-point standard curves plus the non-template control (NTC) were run for each sample to test the relative expression level. Quantitative real-time PCR was conducted, as follow: 2 minutes at 50 °C, 5 minutes at 95 °C, 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C, and 2 cycles of 15 seconds at of 95 °C and 65 °C for 1 minute for the melt curve. All the analyses, including the threshold cycle (Ct) were automatically established using the default settings. Data was analyzed and transformed using the standard curve and the Applied Biosystems 7500 software. Data were then normalized with the geometric mean of the internal control Genes (ICG).

Quantitative Real-Time PCR Performance

Efficiency of qPCR amplification for each gene was calculated using the standard curve method [Efficiency = 10(–1/slope)]. Relative mRNA abundance among measured genes was calculated, using the inverse of qPCR efficiency raised to ΔCt (gene abundance = 1/EΔCt, where

ΔCt = Ct of tested gene – geometric mean Ct of 3 internal control genes). Overall mRNA abundance for each gene was calculated using the median ΔCt, and overall percentage of relative mRNA abundance was computed from the equation: 100 × mRNA abundance of each individual gene / sum of mRNA abundance of all the genes investigated (Table 3.2).

Statistical Analysis

Statistical analysis of collected data was performed using SAS (v9.4 Institute Inc., Cary, NC). Linear mixed models using the MIXED procedure (Littell et al., 1998) were constructed to analyze relative mRNA expression of TLR and inflammatory cytokines. For every TLR and inflammatory cytokine analyzed the model contained the fixed effects of treatment, tissue, and their interaction. Pig was considered as random effect. Least squares means were calculated and are presented with the respective standard error (SEM). Degrees of freedom were estimated by using the Kenward-Roger method (Littell et al., 1998) in the model statement. Residual distribution for each variable was assessed for normality and homoscedasticity. Residual distribution for TLRs and inflammatory cytokines were found lacking of normality and homoscedasticity. Therefore, data were transformed using the Box-Cox procedure of SAS. The log was found to be best transformation for TLRs and inflammatory cytokines data. Statistical significance was declared at $P \leq 0.05$ and trends toward significance effects were noted when $0.05 < P \leq 0.10$.

RESULTS

Relative mRNA Expression of TLR in the GI Tract of Young Pigs

Pigs remained clinically healthy throughout the study and did not reveal diarrhea. Tissues from intestinal mucosa in jejunum, jejunum lymph node, jejunum peyer‘s patches, ileum, ileum lymph node, ileum peyer’s patches, colon, and colon peyer’s patches were used to assess relative mRNA expression of TLR2, TLR4, and TLR10. The mRNA expression of the aforementioned TLR was detectable in all tested tissues. However, treatment did not have effect ($P > 0.05$) on their relative mRNA expression (Table 3.3). Relative mRNA expression of TLR2, TLR4, and TLR10 changed ($P < 0.01$) among biogeographical locations in the GI tract of young pigs (Table 3.4). Higher expression ($P < 0.01$) of TLR2 was observed in jejunum lymph nodes, ileum, ileum lymph nodes, ileum peyer’s patches, colon, and colon lymph nodes tissues, whereas lower expression occurred in jejunum and jejunum peyer ‘s patches (Figure 3.1). Similarly, TLR4 expression was higher ($P < 0.01$) in jejunum lymph nodes, ileum, ileum lymph nodes, ileum peyer’s patches, and colon tissues, whereas lower expression was observed in jejunum, jejunum peyer ‘s patches, and colon lymph nodes (Figure 3.2). Expression of TLR10 was higher ($P < 0.01$) in ileum, ileum lymph nodes, and ileum peyer’s patches tissues, whereas lower expression occurred in jejunum, jejunum peyer‘s patches, colon, and colon lymph nodes (Figure 3.3). Constantly higher expression of TLR’s were observed in ileum and colon.

Relative mRNA Expression of Immunoregulatory Cytokines in the GI Tract of Young Pigs

The mRNA expression of anti-inflammatory cytokines TNFα, IFNγ, IL4, and IL10 was detectable in all tested tissues. However, treatment did not have effect ($P > 0.05$) on their relative mRNA expression (Table 3.3). The mRNA expression of TNFα, IFNγ, IL4, and IL10 changed ($P <0.05$) among biogeographical locations in the GI tract of young pigs (Table 3.4). Higher ($P <$

0.01) mRNA expression of TNFα was observed in jejunum lymph nodes, jejunum peyer's patches, ileum, ileum lymph nodes, ileum peyer's patches, colon, and colon lymph nodes tissues, whereas lower expression occurred in jejunum (Figure 3.4). Likewise, IFNγ and IL4 expression were higher ($P < 0.01$) in most sections of the GI tract, except in jejunum, of young pigs (Figure 3.5 and Figure 3.6, respectively). Higher ($P = 0.02$) expression of IL10 was observed in jejunum, jejunum lymph nodes, ileum, ileum lymph nodes, ileum peyer's patches, and colon lymph nodes tissues, whereas lower expression occurred in jejunum peyer's patches and colon (Figure 3.7).

Overall, our data suggested that expression of TLR (2, 4, and 10) and cytokines (TNFα, IFNγ, IL4, and IL10) were more consistent in ileum peyer's patches and lymph nodes tissues. Thus, revealing novel information about the distribution and expression patterns of these in the GI tract of the piglet.

DISCUSSION

Gastrointestinal diseases bring great economic loss to the swine industry. The intestinal epithelium (IEC), peyer's patches, and gut associated lymphoid tissues all work together to withstand infections. The innate immune response included the toll-like receptors play a crucial role in the detection of microbes and promotion of immunoregulatory cytokines (Uddin et al., 2013). Recognition of microbial products by toll-like receptors leads to an inflammatory response (Fukata and Abreu, 2008), there are more than ten TLR molecules that recognize microbial products. These TLR's are divided according to their localization on the cell and the ligand they recognize (Uenishi and Shinkai, 2009). TLR 2 and TLR 4 are expressed on the cell surface; TLR 4 recognizes lipopolysaccharides (LPS) and TLR 2 recognizes peptidoglycans and

lipoteichoic acid found in gram positive bacteria. TLR10 ligand has not yet been identified although research suggests it is associated with the signaling protein myeloid differentiation primary response gene 88 (MYD88). For certain TLR's MY88 molecules promote inflammatory cytokines such as TNFα. For TLR 4 signaling is dependent on a TIR (toll-IL-1 receptor) domain that induces transcription of type I IFNs. TLRs are necessary to promote a balanced immune response. As part of a larger study assessing microbial populations in newborn pigs, and supporting the idea that the GI microbiota is involved in the development and regulation of the immune system, this study sought to establish a baseline for mucosal immune gene expression in young pigs reared in a cross-fostering model. Quantitative real-time PCR is one of the most widely used techniques for reliable quantification of mRNA (Derveaux et al., 2010). In the present study, qPCR was used to assess the expression of important pattern-recognition receptors and immunoregulatory cytokines in the gut of newborn pigs. Three housekeeping genes were utilized and qPCR efficiency was measured by standard curves with trustworthy correlation coefficient. Therefore, we are confident that the results obtained are reliable. In general, our results revealed that TLR were expressed higher in ileum and ileum associated lymph tissues of young pigs (21 days). Higher mRNA expression was seen in ileum peyer's patches and lymph nodes. According to Kelly and Mulder (2012), peyer's patches are a crucial region where the immune system is stimulated and regulated, comprised of structured lymphoid tissues that directly sample antigens from the intestine. Additionally, unlike the colon, ileum has a much thinner mucus layer, thus enabling microbe-epithelial interaction. The overall expression of TLR was low, in a study performed by Kitazawa et al. (2008) expression levels of TLR family are regulated by microbiota established after birth, these microbiota is responsible for promoting a balanced immune response. In a similar way, Hrncir et al. (2008) agreed that the interaction of

microbial molecular patterns with pattern recognition receptors (TLRs) in dendritic cells located in peyer's patches results in a relocation to the mesenteric lymph nodes and later presentation to T cells via a MHC class II-antigen complex, which activates and differentiates T cells and promotes production of cytokines. In our study, animals were 21 days old and therefore no excessive inflammation was promoted since microbial colonization is commencing. Moreover, pigs were healthy and no intestinal disease was revealed, thereby, no tissue damage occurred. Akira and Takeda (2004) highlighted the association between tissue damage and TLR4 expression. It is possible, therefore that microbial establishment and maturation in the gut is crucial for the regulation of the immune system. Additionally, according to Cheng et al. (2015) TLRs are highly expressed 6 – 12 weeks after birth. Likewise, Uddin et al. (2011) asserted that TLR2 mRNA expression was augmented in full-grown pigs. Tohno et al. (2006) found increased expression of TLR2 and TLR9 in adult GALT. Thus, it can be suggested that the overall low expression of TLR obtained in our study can be attributed to the age of the young pig and a low promotion of expression by the microflora at that early age. Kogut and Arsenault (2016) performed a study in broiler chickens and found regional differences in the microbiome, associated with regional differences in immune gene expression. A negative correlation between pro-inflammatory cytokine genes and the presence of Firmicutes was established as well as a positive correlation between pro-inflammatory cytokine genes and the presence of *Proteobacteri*a. In our study colon had a lower abundance of *Proteobacteria* and a lower expression of cytokines and TLR's especially in that of treatment 3. Colon mRNA expression was low compared to ileum or jejunum. Higher expression of *Proteobacteria* was seen on day 21 compared to day 0, which is in accord to previous authors that promotion of expression by the microflora increases with age. Although we did not measure immune gene expression over time,

this is definitely something to look into. Although we did not associate the microbiota found with immune gene expression, we did find regional differences in the microbiome and regional differences in immune gene expression.

Our observation that the abundance of TLR mRNA expression is different between tissues is consistent with previous studies (Sang et al., 2008; Uddin et al., 2011; McDermott and Huffnagle, 2014). A possible explanation is that each tissue has different function. Nutrient absorption occurs in the small intestine and water absorption in the large intestine, this particular functionality is mainly attributed to the microbiota composition which may possibly be the underlying element that regulates the expression of TLR and overall immunological homeostasis. For this reason, it is important to measure gene expression at different time points in each tissue and microbiota assessment as well, to determine the variation of mucosal immunity and microbiota shift of the pigs as it ages.

CONCLUSION

The present study confirmed the mRNA expression of TLRs and inflammatory cytokines in gastrointestinal tissues and gut-associated lymphoid tissues. Differential expression patterns of TLRs and anti-inflammatory cytokines in tissues revealed that TLRs recognition and immune response is not only bound to immune cells, but also to non-immune cells. TLRs may play a role in immune modulation and higher exposure to microbes could potentially create a definitive balance between pathogen and commensal bacteria in the gut. Although mRNA expression confirms the presence of TLRs and inflammatory cytokines, the functional importance of these needs to be further investigated. For future studies establishing the relationship between

microbiota and specific genera with immune gene expression are necessary to elucidate hey microbes crucial in immune modulation. In addition, the immune mechanisms at a cellular level are needed to understand TLR recognition and inflammatory response at different histological layers.

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Table 3.1. Primers used for reverse transcriptase-PCR of swine cytokines

Gene name	Forward (5'→3')	Reverse (5'→3')	GenBank accession
swTNFα swine porcine tumor necrosis alpha	GCCCTTCCACCAACGTTTTC	CCCAGGTTGCATCCAGGAAT	NM_214022.1
swIFNγ swine interferon gamma	GATCCAGCGCAAAGCCATCA	TCTGGCCTTGGAACATAGTCTG	NM_213948.1
swIL4 swine interleukin 4	CTCCCAACTGATCCCAACCC	TGCACGAGTTCTTTCTCGCT	NM_214123.1
swIL10 swine interleukin 10	GTGCTCTCCCTGCCAAAGAT	AATGGGGAGGCCTCACTGAA	NC_010451.3
swTLR2 swine toll-like receptor 2	GGAATGTACCTTTGGCCCGA	ACGGTGTGCTGCAAGGTAAT	NC_010450.3
swTLR4 swine toll-like receptor 4	GCTTTTACCACTATCCAGAGCA	ATTGGCATCCCGCTCAGTTT	NC_010443.4
swTLR10 swine toll-like receptor 10	CCTCGGAGATAGGCATGCTG	TTGGGTTGGGACCCTATTCC	NC_010450.3
swGAPDH swine Glyceraldehyde 3-phosphate dehydrogenase	CTGCTCGGGAAAACCACACT	TGTGTTGGGGGATCGAGTTG	NC_010447.4
swβ-2M swine beta-2 microglobulin	CAACCACTTTTCACACCGCT	TGTGATGCCGGTTAGTGGTC	NM_213978.1
swβ-actin swine beta-actin	AGGGTCAGGATGCCTCTCTT	GGCTTCCTTTGTCCCCAATC	NC_010445.3

Table 3.2. Median cycle threshold (Ct) of qPCR, slope, coefficient of determination of the standard curve (R2), and efficiency of amplification

Gene name	Median Ct[1]	Slope[2]	(R^2)[4]	Efficiency[3]	Relative mRNA Abundance[4]
TLR2	22.62063789	-3.48765	0.99655	1.935200729	44.82404517
TLR4	25.06811333	-3.68455	0.99545	1.888114812	10.62742834
TLR10	26.88232994	-3.63135	0.99115	1.895296586	2.688739481
TNFα	25.34217644	-3.6763	0.98832	1.870736507	8.977664625
IFNγ	27.65718079	-3.6114	0.99655	1.891911969	2.853841436
IL4	23.1246	-34917	0.99756	0.933721936	22.20647484
IL10	26.43345	-395595	0.985632	1.893267785	7.821806101

[1] Ct = median cycle threshold which is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceed background level); and the amount of target nucleic acid in the sample is inversely correlated to Ct cycles (i.e., the greater the amount of target nucleic acid the lower the Ct cycles will be required).
[2] Slope of the 5-point standard curve.
[3] Efficiency of amplification [E=10(−1/slope)].
[4] Relative mRNA abundance was calculated as percentage of (1/EΔCt) specific gene in the sum (1/EΔCt) all genes.

Table 3.3. Least square means and associated SEM for expression of genes related to inflammation and pattern recognition

	Treatment[1]			SEM[2]	*P*[3]		
Gene	1	2	3		Trt	Tissue	Trt × Tissue
TLR2	1.41	1.58	0.85	0.33	0.45	<0.01	0.85
TLR4	0.24	0.32	0.45	0.45	0.33	<0.01	0.001
TLR10	1.63	1.42	1.20	0.49	0.65	<0.01	0.08
TNFα	1.62	1.18	0.74	0.87	0.54	<0.01	0.19
IFNγ	1.57	1.44	0.71	0.93	0.62	<0.01	0.23
IL4	1.40	1.49	1.16	0.38	0.73	0.01	0.34
IL10	2.03	1.84	1.18	0.66	0.74	0.02	0.03

[1] Treatment: 1 = litter received colostrum and post-colostrum milk feeding from their own dam; 2 = litter exchanged at birth to receive colostrum from a foster dam and then returned to their own dam for post-colostral milk feeding for the subsequent days; and 3 = litter exchanged at birth to receive colostrum and post-colostrum milk feeding from a foster dam, they remained with the foster dam for the subsequent days.
[2] Greatest SEM.
[3] Trt = 1, 2, and 3; tissue = jejunum, jejunum lymph node, jejunum peyer's patches, ileum, ileum lymph node, ileum peyer's patches, colon, and colon lymph node; trt × tissue = interaction of treatment and tissue.

Table 3.4. Least square means and associated SEM for expression of genes related to inflammation and pattern recognition

	Tissue[1]								SEM[2]	*P*[3]		
Gene	JEJ	JLN	JPP	ILE	ILN	IPP	COL	CLN		Trt	Tissue	Trt × Tissue
TLR2	0.55^{c}	1.60^{abc}	0.55^{ac}	1.95^{ab}	1.50^{a}	1.26^{ab}	1.72^{ab}	1.13^{abc}	0.40	0.45	<0.01	0.85
TLR4	0.57^{b}	1.24^{ab}	0.96^{b}	1.90^{ab}	1.70^{a}	0.86^{ab}	1.19^{ab}	0.65^{b}	0.37	0.33	<0.01	<0.01
TLR10	0.40^{c}	2.33^{ab}	1.11^{bc}	1.93^{a}	2.09^{a}	1.65^{ab}	0.60^{c}	1.24^{abc}	0.49	0.65	<0.01	0.08
TNFα	0.45^{b}	1.12^{a}	1.68^{ab}	1.58^{a}	1.54^{a}	1.30^{a}	0.96^{ab}	0.81^{ab}	0.70	0.54	<0.01	0.19
IFNγ	0.49^{b}	1.51^{a}	2.36^{ab}	0.60^{ab}	1.75^{a}	1.16^{a}	1.23^{a}	0.81^{a}	0.83	0.62	<0.01	0.23
IL4	0.70^{b}	1.18^{ab}	1.04^{ab}	1.15^{ab}	1.69^{ab}	1.71^{a}	1.86^{ab}	1.46^{ab}	0.46	0.73	0.01	0.34
IL10	1.12^{ab}	1.92^{ab}	1.81^{b}	2.59^{a}	2.16^{ab}	1.83^{ab}	0.92^{b}	1.12^{ab}	0.77	0.74	0.02	0.03

[1] JEJ = jejunum, JLN = jejunum lymph node, JPP = jejunum peyer's patches, ILE = ileum, ILN = ileum lymph node, IPP = ileum peyer's patches, COL = colon, and CLN = colon lymph node.
[2] Greatest SEM.
[3] Treatment: 1 = litter received colostrum and post-colostrum milk feeding from their own dam, 2 = litter exchanged at birth to receive colostrum from a foster dam and then returned to their own dam for post-colostral milk feeding for the subsequent days, and 3 = litter exchanged at birth to receive colostrum and post-colostrum milk feeding from a foster dam, they remained with the foster dam for the subsequent days; tissue = jejunum, jejunum lymph node, jejunum peyer's patches, ileum, ileum lymph node, ileum peyer's patches, colon, and colon lymph node; trt × tissue = interaction of treatment and tissue.

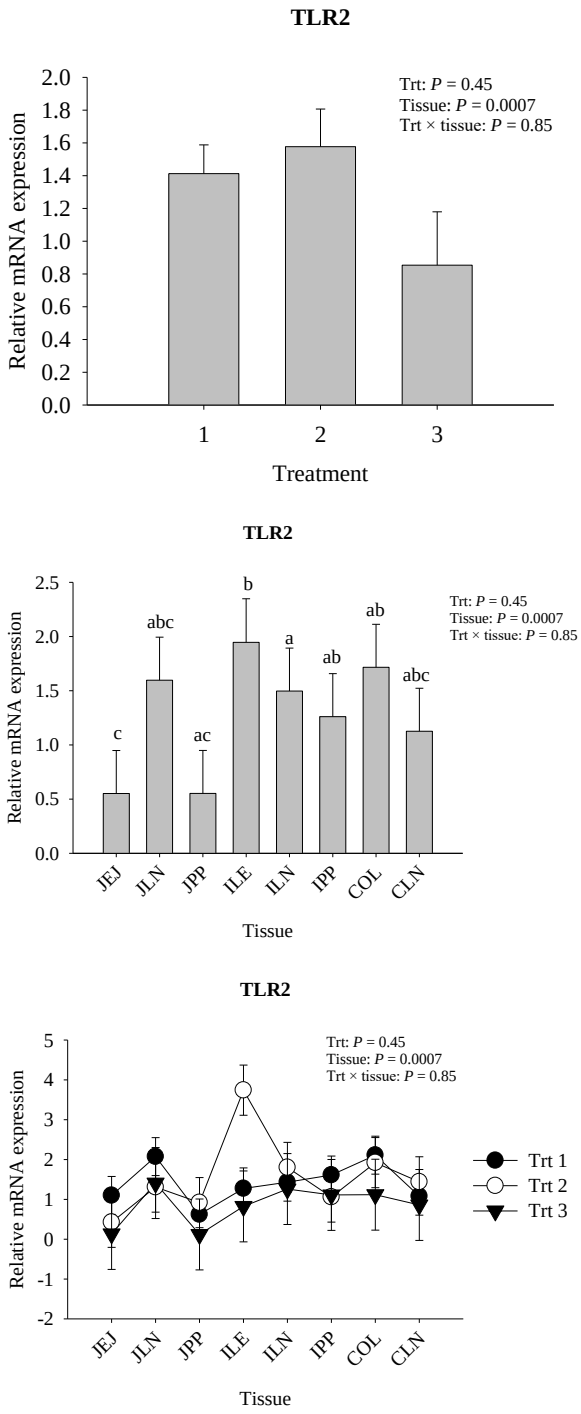

Figure 3.1. Relative mRNA expression of toll-like receptor 2 (TRL2) in the jejunum (JEJ), jejunum lymph node (JLN), jejunum peyer's patches (JPP), ileum (ILE), ileum lymph node (ILN), ileum peyer's patches (IPP), colon (COL), and colon lymph nodes (CLN) of young pigs. Treatment: $P = 0.40$; tissue: $P < 0.01$; and interaction of treatment and tissue: $P = 0.85$.

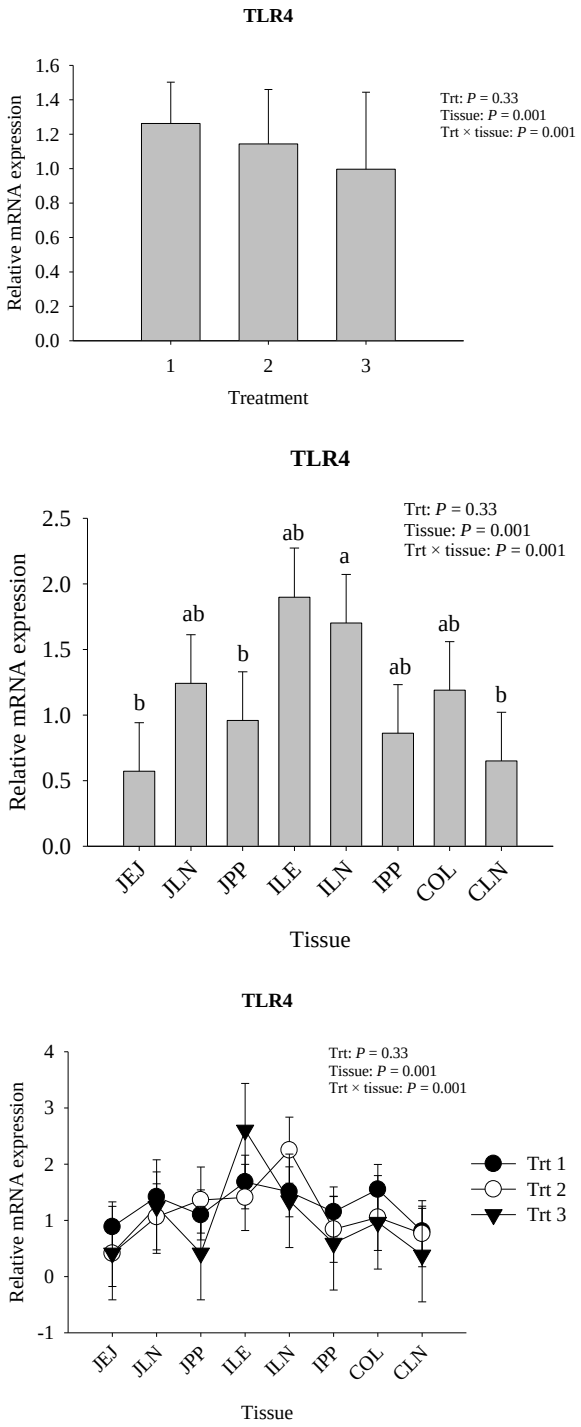

Figure 3.2. Relative mRNA expression of toll-like receptor 4 (TRL4) in the jejunum (JEJ), jejunum lymph node (JLN), jejunum peyer's patches (JPP), ileum (ILE), ileum lymph node (ILN), ileum peyer's patches (IPP), colon (COL), and colon lymph nodes (CLN) of young pigs. Treatment: $P = 0.33$; tissue: $P < 0.01$; and interaction of treatment and tissue: $P < 0.01$.

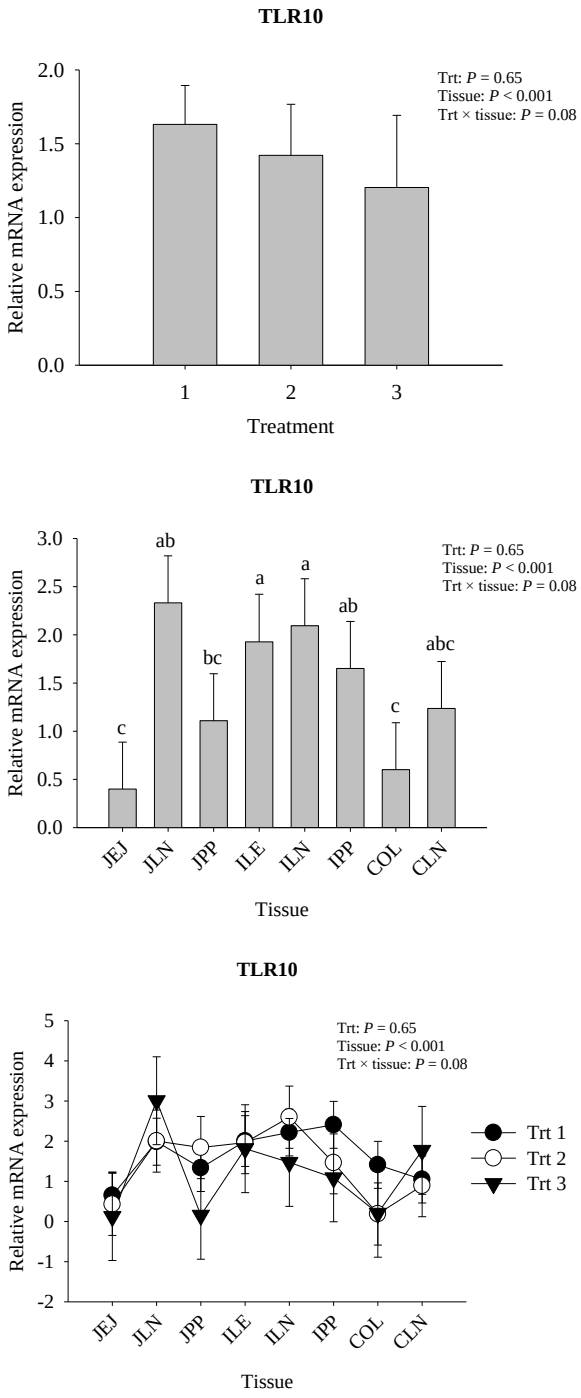

Figure 3.3. Relative mRNA expression of toll-like receptor 10 (TRL10) in the jejunum (JEJ), jejunum lymph node (JLN), jejunum peyer's patches (JPP), ileum (ILE), ileum lymph node (ILN), ileum peyer's patches (IPP), colon (COL), and colon lymph nodes (CLN) of young pigs. Treatment: $P = 0.49$; tissue: $P < 0.01$; and interaction of treatment and tissue: $P = 0.08$.

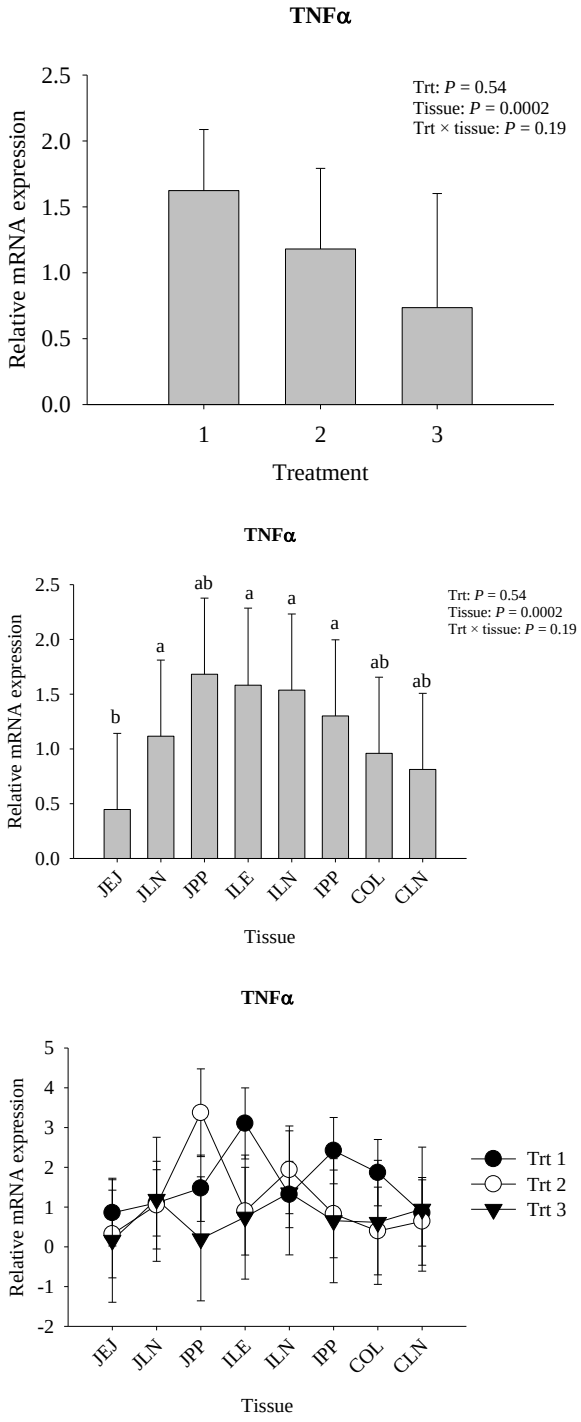

Figure 3.4. Relative mRNA expression of tumor necrosis factor alpha (TNFα) in the jejunum (JEJ), jejunum lymph node (JLN), jejunum peyer's patches (JPP), ileum (ILE), ileum lymph node (ILN), ileum peyer's patches (IPP), colon (COL), and colon lymph nodes (CLN) of young pigs. Treatment: $P = 0.54$; tissue: $P < 0.01$; and interaction of treatment and tissue: $P = 0.19$.

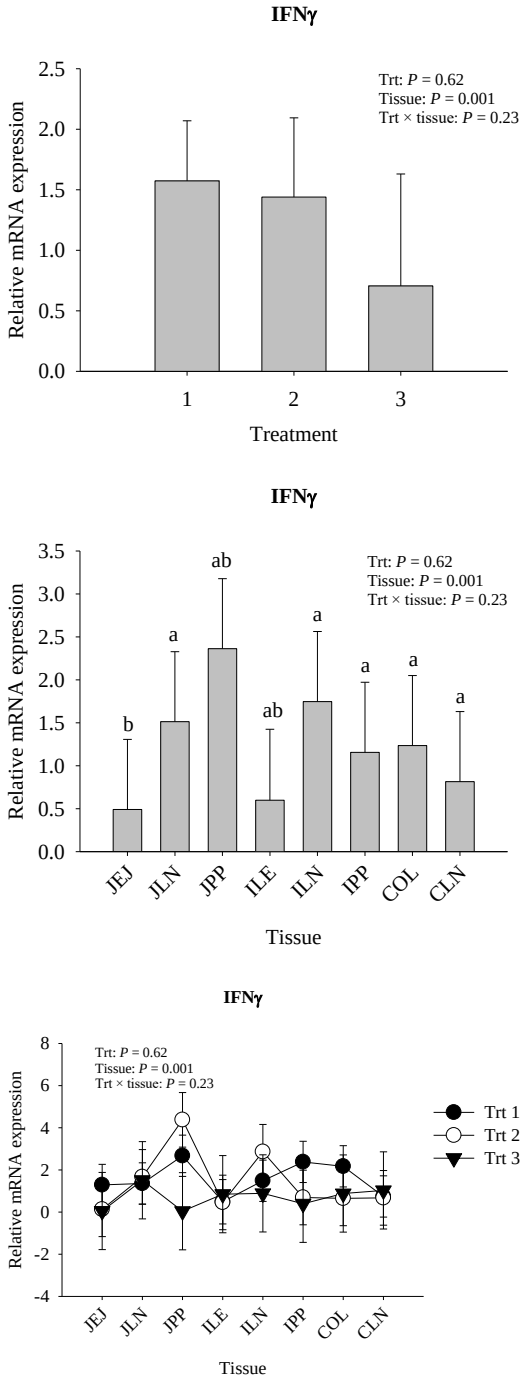

Figure 3.5. Relative mRNA expression of interferon gamma (IFNγ) in the jejunum (JEJ), jejunum lymph node (JLN), jejunum peyer's patches (JPP), ileum (ILE), ileum lymph node (ILN), ileum peyer's patches (IPP), colon (COL), and colon lymph nodes (CLN) of young pigs. Treatment: $P = 0.62$; tissue: $P < 0.01$; and interaction of treatment and tissue: $P = 0.23$.

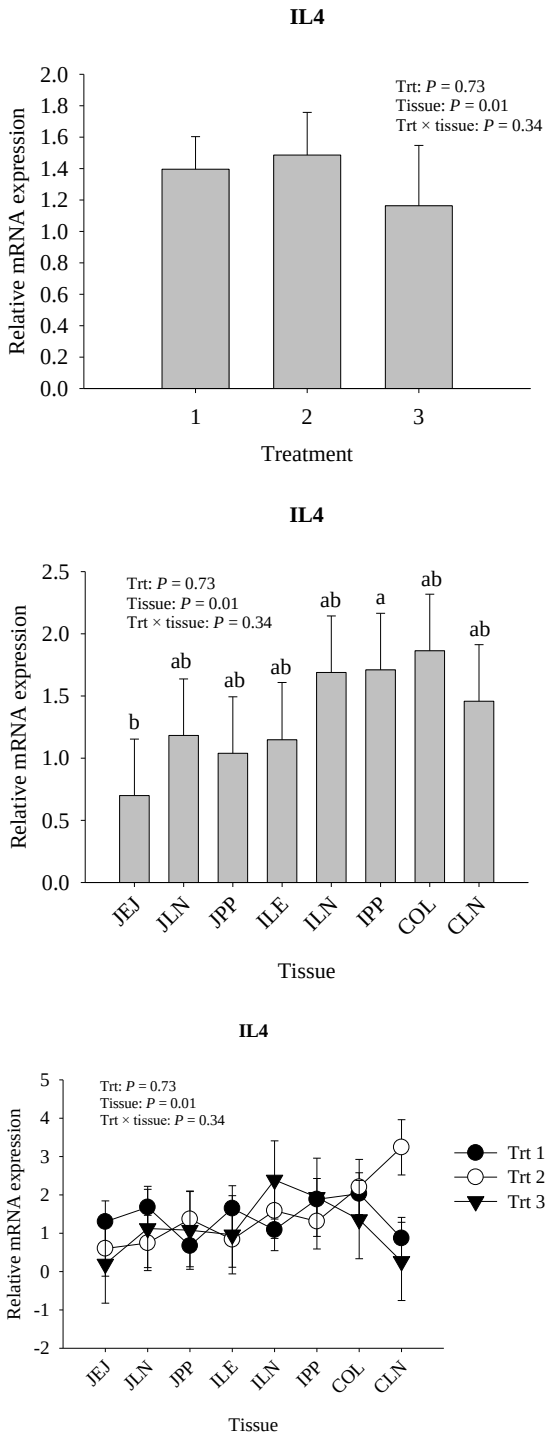

Figure 3.6. Relative mRNA expression of interleukin 4 (IL4) in the jejunum (JEJ), jejunum lymph node (JLN), jejunum peyer's patches (JPP), ileum (ILE), ileum lymph node (ILN), ileum peyer's patches (IPP), colon (COL), and colon lymph nodes (CLN) of young pigs. Treatment: $P = 0.73$; tissue: $P = 0.01$; and interaction of treatment and tissue: $P = 0.34$.

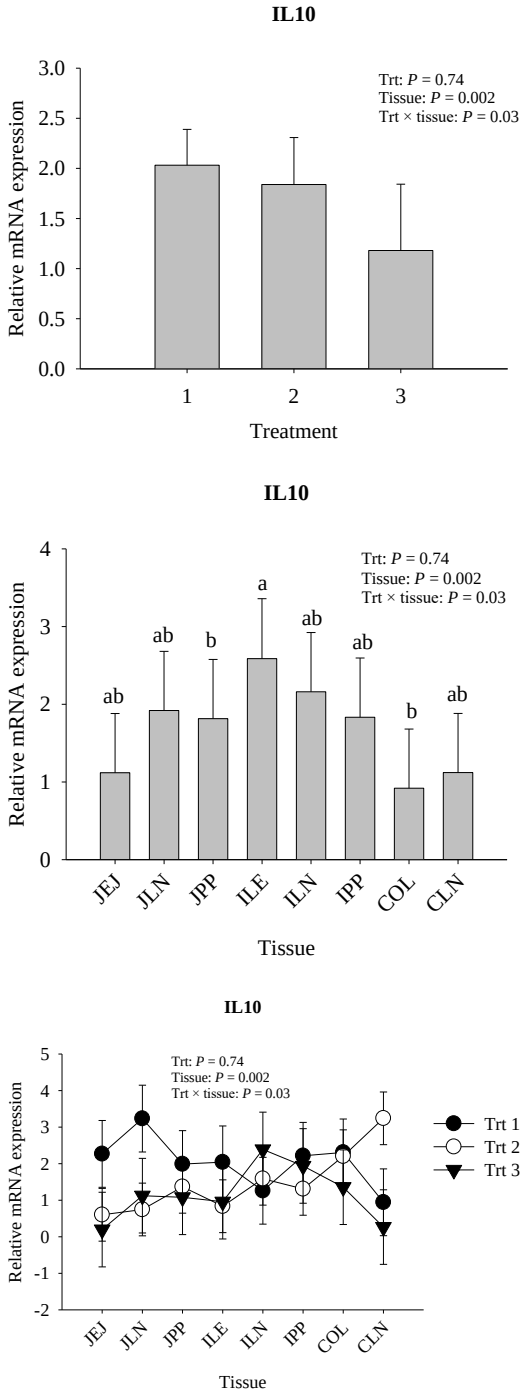

Figure 3.7. Relative mRNA expression of interleukin 10 (IL10) in the jejunum (JEJ), jejunum lymph node (JLN), jejunum peyer's patches (JPP), ileum (ILE), ileum lymph node (ILN), ileum peyer's patches (IPP), colon (COL), and colon lymph nodes (CLN) of young pigs. Treatment: $P = 0.74$; tissue: $P = 0.02$; and interaction of treatment and tissue: $P = 0.03$.